The use of *Saccharomyces cerevisiae* as a model system to study the mechanism of action of chemical antioxidants

Chuleeporn Chumnanka

Submitted for the degree of Doctor of Philosophy

Heriot-Watt University

School of Life Sciences

June 2016

ABSTRACT

Butylated hydroxyanisole (BHA) is a synthetic substance commonly used as dietary antioxidant which plays an important role in protecting foods from oxidation. It has been reported that this compound caused an increase in glutathione-s-transferase specific activity, which is responsible for resistance to oxidative stress, i.e., there was some evidence that it could act as a pro-oxidant. In the present study, BHA was found to be toxic towards yeast and this was investigated further by determining its effect on the viability of a variety of yeast mutants lacking key genes required for resistance to oxidative stress. The results showed that the cell viability of the mutants was not significantly different from the wild type parental strains. This suggests that BHA toxicity was unlikely to involve reactive oxygen species (O_2^- and H_2O_2) and might not directly involve the classical oxidant stress responses found in yeast cells. Interestingly, the pre-treatment of yeast cells with low concentrations of BHA (0.2 mM) and subsequent exposure to higher concentrations of either BHA or hydrogen peroxide (H_2O_2) resulted in pre-treated yeast cells becoming more resistant to both toxic levels of BHA and H_2O_2 than non pre-treated cells. These findings indicated that low level of BHA could induce an adaptive response to BHA and induce cross-protection against H_2O_2. A genetic approach was adopted to identify genes involved in this process, involving the isolation and characterisation of BHA sensitive mutants. The results demonstrated that the yeast genes *TYR1*, *KRE6* and *GPH1* play role in the cells response towards BHA.

ACKNOWLEDGEMENTS

I wish to thank Dr. Derek J. Jamieson, my supervisor for his advice, help and critical reading of this thesis. Also, I would like to thank Qianmei Zhai and Meredith Hamilton for their help in some parts of my experiments. This study was supported by Ministry of Science and Technology, The Royal Thai Government, Thailand.

ACADEMIC REGISTRY
Research Thesis Submission

Name*:*	Chuleeporn Chumnanka		
School/PGI:	Life Sciences		
Version: *(i.e. First, Resubmission, Final)*	Final	Degree Sought (Award **and** Subject area)	PhD (Brewing)

Declaration

In accordance with the appropriate regulations I hereby submit my thesis and I declare that:

1) the thesis embodies the results of my own work and has been composed by myself
2) where appropriate, I have made acknowledgement of the work of others and have made reference to work carried out in collaboration with other persons
3) the thesis is the correct version of the thesis for submission and is the same version as any electronic versions submitted*.
4) my thesis for the award referred to, deposited in the Heriot-Watt University Library, should be made available for loan or photocopying and be available via the Institutional Repository, subject to such conditions as the Librarian may require
5) I understand that as a student of the University I am required to abide by the Regulations of the University and to conform to its discipline.

* *Please note that it is the responsibility of the candidate to ensure that the correct version of the thesis is submitted.*

Signature of Candidate*:*	ชุลีพร ชำนาญค้า	Date:	22/06/2016

Submission

Submitted By *(name in capitals):*	
Signature of Individual Submitting:	
Date Submitted:	

For Completion in the Student Service Centre (SSC)

Received in the SSC by *(name in capitals):*			
Method of Submission *(Handed in to SSC; posted through internal/external mail):*			
*E-thesis Submitted (**mandatory for final theses**)*			
Signature:		Date:	

Please note this form should bound into the submitted thesis.

Updated February 2008, November 2008, February 2009, January 2011

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABBREVIATIONS AND SYMBOLS

≈	approximately
%	percentage
Δ	deletion
Amp	Ampicillin
ARE	antioxidant response element
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
bp	base pair
ºC	degree celsius
$CaCl_2$	calcium chloride
CAT	catalase
EMS	ethylmethane sulfonate
GPX	glutathione peroxidase
GR	glutathione reductase
GST-Ya	glutathione S-transferase Ya subunit gene
g	gram
g/l	gram per liter
H_2O_2	hydrogen peroxide
his	histidine
kbp	kilo base pair
KCl	potassium chloride
leu	leucine

lys	lysine
M	molar
$MgSO_4.7H_2O$	magnesium sulfate
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
mg	milligram
mg/kg	milligram per kilogram
mg/ml	milligram per milliliter
ml	milliliter
mM	milimolar
mm	millimeter
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$Na_2HPO_4.7H_2O$	sodium monohydrogen phosphate heptahydrate
$Na_2HPO_4.H_2O$	disodium hydrogen phosphate
NaOH	sodium hydroxide
nm	nanometer
OD_{600}	optical density at 600 nm
ONPG	o-nitrophenyl-β-D-galactoside
PEG	polyethylene glycol
phe	phenylalanine
r.p.m	revolutions per minute
ROS	reactive oxygen species
SGD	*Saccharomyces* genome database
SOD	superoxide dismutase

TBHQ	butyl hydroquinone
ura	uracil
µg/ml	microgram per milliliter
µl	microliter
µM	micromole
µm	micron
w/v	weight volume ratio
YEp	yeast episomal plasmid

CHAPTER 1 - INTRODUCTION

1.1 Reactive oxygen species (ROS)

Reactive Oxygen Species (ROS) are generated during normal metabolic process of all aerobic cells. In addition, ROS can be formed when the cells are exposure to radical-generating compounds (Allen and Tresini, 2000; Matés, 2000; Bednarska, et al., 2008; Greetham and Grant, 2009 and Sen and Chakraborty, 2011). ROS consist of hydroxyl radicals (HO^-), hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-). When levels of ROS in cells is higher than antioxidant activities, oxidative stress arises, which can damage cell structures and be the cause of serious diseases like cancer, cardiovascular and dysfunction.

1.2 Antioxidants

Antioxidants are defined as natural or chemical substances which at low concentration can scavenge free radicals and delay or inhibit oxidative processes (Sies, 1993; Halliwell et al., 1995; Sen and Chakraborty, 2011 and Nimse and Pal, 2015). They play a vital role in preventing oxidative stress of cells. Sen and Chakraborty (2011) reported that antioxidants could decrease reactive oxygen species (ROS), free radical levels and toxicity by several different mechanisms. For instance, the first mechanism involves enzymatic destruction/conversion of the reactive compounds to less harmful or – reactive compound. The enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). The second is binding or inactivation of metal ions to prevent ROS generation by the Haber-Weiss reaction. The Haber-Weiss reaction is a reaction that can generate hydroxyl radical ($HO^{\cdot}$) from an interaction between hydrogen peroxide (H_2O_2) and superoxide ($O^{\cdot}{}_2^-$) (Kehrer, 2000). The third is scavenging free radicals and destroying ROS. The final mechanism is quenching of ROS which is antioxidants react with oxygen radicals. Antioxidants such as β-carotene can deactivate singlet oxygen ($^1O_2$) by energy transfer or charge transfer (Larson, 1995 and Choe and Min, 2009). Antioxidants classified in many groups which are shown in Table 1.1.

A number of chemical antioxidants; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and butyl hydroquinone (TBHQ) are widely used in many products. For example, BHA and BHT have been used in food products, animal feed, cosmetics and petroleum products (IARC, 1986; Williams, et al., 1999 and Anonymous,

2011). These antioxidants act as preservatives to prevent lipid peroxidation. Williams, et al. (1999) revealed that the US Food and Drug Administration (FDA) permitted the use of BHA and BHT as food additive. The levels of BHA, BHT and TBHQ which are allowed in foods are 100-400 mg/kg, 75-400 mg/kg and 100-400 mg/kg, respectively (CODEX, 2011).

Table 1.1 Classification of antioxidants and their regulation functions

Antioxidants categories	Regulation functions
A. Classification based upon their nature	
1. Enzymatic antioxidant	
Superoxide dismutase (SOD)	Dismutation of superoxide to hydrogen peroxide (H_2O_2)
Catalase (CAT)	Dismutation of H_2O_2 to molecular oxygen and water
Glutathione peroxidase (GPX)	Reduction of H_2O_2 and other hydroperoxides, lipid peroxides, lipoxygenase products
Glutathione reductase (GR)	Reduction of low molecular weight disulfides
2. Non-enzymatic antioxidant	
2.1 Metabolic antioxidants	
Reduced glutathione (GSH)	Reducing H_2O_2 to water with the formation of glutathione disulphide (GSSG)
	It reacts with superoxide anion, hydroxyl, and alkoxyl radical directly by a radical transfer process and inhibits tissue damage.

Source: Sen and Chakraborty (2011)

Table 1.1 Classification of antioxidants and their regulation functions (continue)

Antioxidants categories	Regulation functions
Coenzyme Q_{10}	It inhibits lipid peroxidation, reduces mitochondrial oxidative stress and can also able to recycle vitamin E.
Uric acid	It is a scavenger of singlet oxygen and radicals like superoxide anion, hydroxyl, and alkoxyl radical and binds transition metal
Transferrine	It bind free iron salts, which can lead to the generation of reactive oxygen species
2.2 Nutrient antioxidants	
Vitamin E	Direct scavenging of superoxide, hydroxyl radicals, upregulation of antioxidation enzymes, breaks lipid peroxidation chain reactions.
Vitamin C	Scavenges superoxide, hydroxyl radicals, neutralize oxidants from stimulated neutrophils, regenerates vitamin E
Carotenoids	Antioxidant activity of carotenoids is based on their singlet oxygen quenching activity.
Flavonoids	Flavonoids capable scavenge hydroxyl radical, superoxide anions and lipid peroxy radicals.

Source: Sen and Chakraborty (2011)

Table 1.1 Classification of antioxidants and their regulation functions (continue)

Antioxidants categories
B. Classification based upon source
1. Endogenous Antioxidants Bilirubin, glutathione, lipoic acid, N-acetyl cysteine, NADPH and NADH, ubiquinone (coenzyme Q_{10}), uric acid, enzymes (SOD, CAT, GPX, GR)
2. Metal Binding Proteins Albumin (copper), ceruloplasmin (copper), metallothionein (copper), ferritin (iron), myoglobin (iron), transferring (iron)
C. Classification based mechanism of action
1. Catalytic systems to neutralise or divert ROS SOD, CAT, GPX
2. Binding/inactivation of metal ions prevents production of ROS by Haber-Weiss Ferritin, caeruloplasmin, catechins
3. Self suicidal and chain breaking antioxidants scavenge, destroy ROS Vitamin C, Vitamin E, uric acid, glutathione, flavonoids
4. Quenching ROS, chemical traps/sinks to absorb energy Carotenoids, anthocyanidins

Source: Sen and Chakraborty (2011)

1.2.1 Mode of action

With regard to mode of action, synthetic antioxidants including BHA, BHT, TBHQ and gallates act as free radical terminators are classified as primary antioxidants. In addition, oxygen scavengers (glucose oxidase and sulphites) and chelating agents (iron and copper) are primary antioxidants as well. Verhagen, et al. (1991) reported that BHA and BHT could terminate the continuing lipid autoxidation reaction by acting as radical scavengers. These phenolic antioxidants give a hydrogen atom to lipid radicals and turn them into the more stable molecules. Moreover, there is another pathway namely secondary antioxidants which can stabilize food products. These antioxidants, for example, thiodiopropionic acid and dilauryl theodipropionate are able to break down the hydrogen peroxide formed during lipid oxidation into stable end products (Sen and Chakraborty, 2011)

1.2.2 Effect of butylated hydroxyanisole (BHA)

It has been reported that chemical antioxidants have shown both anticarcinogenic and carcinogenic effects (Williams, et al., 1999 and Kahl, 1984). At low concentration, BHA and BHT appear to pose no cancer risk due to free radical scavenging activity and these compounds may also perform anticarcinogenic properties at the levels used (Williams, et al., 1999).

Also, several studies found that BHA exhibited no genotoxicity in mammalian cell models. For example, research from the World Health Organization (WHO) reported that BHA showed no genotoxicity in V79 Chinese hamster lung cells, Salmonella, primary hepatocyte culture, adult rat liver and Chinese hamster ovary cells at BHA dose levels around 0.3 mM, 0.01-10 mg, 10^{-5}-1 mg/ml, 0.05-0.1 mg/ml and 1-10^{-3} mg/ml, respectively. They also suggested that the levels of BHA were no toxicological effect in rats was 1,250 ppm (0.125%) in the diet or equivalent to 62.5 mg/kg body weight/day, whereas, the acceptable daily intake for man was approximately 0-0.3 mg/kg body weight (WHO, 1987). Likewise, Moon and Park (2011) illustrated that low concentrations of BHA (150 μM) decreased HeLa cervical cancer cells viability via caspase-dependent apoptosis.

Furthermore, Benson, et al. (1980) and Moldéus, et al. (1982) as cited in Yu, et al. (2000) revealed that BHA represented protective ability because it induced phase II detoxifying enzymes like glutathione S-transferase, quinine reductases and UDP-glucoronosyl transferases. These enzymes could catalyze metabolic of xenobiotic and

carcinogens and also protect cells from oxidative stress. The expression of detoxifying enzymes gene was regulated by the antioxidant response element (ARE), which is found in the promoter of several detoxifying enzyme genes (Matés, 2000). Rushmore, et al. (1991) showed that the ARE has been identified in the rat glutathione S-transferase Ya (GST-Ya) subunit gene and the rat NAD(P)H: quinone reductase gene. They also found that expression of the GST-Ya gene and the quinone reductase gene was activated by hydrogen peroxide and phenolic antioxidants through the ARE. These studies suggest that the ARE may be a part of signal transduction pathway of eukaryotic cells to be able to respond to oxidative stress. Additionally, Prestera, et al. (1993) showed that a 41-bp enhancer element (containing the ARE element) from the upstream region of the mouse GST-Ya gene regulated phase II detoxifying enzymes in response to toxic electrophiles. This implies that the ARE may respond to both oxidants and antioxidants and its precise role in animals is unclear.

In case of carcinogenic properties, some researchers argued that BHA could induce cell toxicity. For example, Kahl, et al. (1989) demonstrated that BHA could actually stimulate superoxide production in rat liver microsomes. Additionally, they found that the BHA metabolite, tert-butylhydroquinone (TBHQ) is more effective than BHA in excess superoxide formation. Verhagen, et al. (1991) reported that BHA may have carcinogenic potential in animals such as rat, hamster and mouse. The target tissue affected by BHA is the forestomach, bladder, glandular stomach, small intestines and oesophagus. They also reported that BHA may induce changes in DNA, leading to initial tumor formation in the rat forestomach. BHA has been shown to induce forestomach squamous cell carcinomas in rodents at high concentrations of over 3,000 ppm (Williams, et al., 1999).

Yu, et al., (2000) revealed that the cytotoxicity of BHA induced apoptosis in rat hepatocytes. This might be due to the interaction of BHA, mitochondria and cytochrome C. It led to the formation of caspase-3, a member of the cysteine-aspartic acid protease family which cleaves cellular proteins such as DNA fragmentation factor (DFF45), structural protein (α-fodrin) and gelsolin, resulting in sending cells down an apoptotic cell death pathway. Porter and Jänicke (1999) also reported that the cleavage of α-fodrin and gelsolin by caspase-3 may lead to morphological changes including membrane blebbing, as a result of cell apoptosis. In addition, Ali and Suzuki (2012) determined the genotoxicity of BHA and TBHQ in multiple mouse organs such as bone marrow, liver, kidney and stomach. They found that BHA induced DNA damage in

stomach, while TBHQ induced DNA stand breaks in liver and kidney cells. This research suggested that BHA and TBHQ might react with DNA indirectly via an ROS mechanism, which induces oxidative damage.

Although many reviews showed positive effects of BHA at low concentrations, BHA produce negative interactions like cytotoxicity, genotoxicity and carcinogenesis at high doses. It appears that the activity of BHA depends upon the levels of BHA, which were used in these experiments. However, while the effects of chemical antioxidants are widely studied by using mammalian cell culture, like hepatoma cell lines, these cell lines may show different results from that of the whole animal or cells not grown in culture. Additionally, results from animal cell models are difficult to interpret due to the fact that the mechanism and metabolism underlying the inducing antioxidant is not clear.

For this reason, it is essential to use another model system, like yeast, which represents eukaryotic organisms in order to understanding the exact mechanism of action of antioxidants to determine whether they directly induce protective enzyme levels or work by virtue of being weak oxidants that cause a mild form of oxidative stress that induces a protective adaptive response. This is because the metabolism and gene regulation of yeast are well understood. The yeast model may also overcome some of the problems which are encountered with animal cell lines.

1.3 Yeast stress response

1.3.1 Adaptive stress response in Saccharomyces cerevisiae

All living cells possess the ability to respond to stressful environmental changes. This ability is known as stress responses, which means cells are able to adjust their metabolism and cellular processes in order to protect the cells against stressful conditions (Hohmann and Mager, 2010). A number of researchers have demonstrated that the yeast cell possesses adaptive responses against several kinds of stress conditions. For instance, pre-treatment of *S. cerevisiae* cells with low concentrations of either oxidants (menadione - a superoxide generating agent or hydrogen peroxide) or hyperosmotic stress, resulted in the yeast cells become more resistant to higher concentrations of the same stress (Jamieson, 1992; Flattery-O' Brien, et al., 1993; Izawa, et al., 1995 and Lu, et al., 2005). Additionally, yeast cells exposed to mild levels of one type of stress could acquire cross-protection against another type of stress. Jamieson (1992) showed that pre-treatment of *S. cerevisiae* with heat shock (23 to

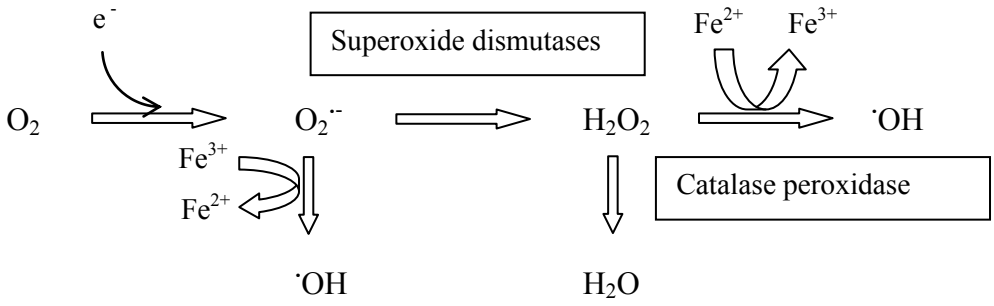
37 ºC) or low concentration of hydrogen peroxide (0.2 mM H_2O_2) could induce protective adaptive response to menadione (Flattery-O'Brien, et al., 1993). However, menadione pre-treated cells did not confer resistance to heat or hydrogen peroxide. This research is consistent with the study of Collinson and Dawes (1992) which demonstrated that *S. cerevisiae* possessed cross-protection between heat stress and hydrogen peroxide, but pre-treatment with hydrogen peroxide did not protect yeast cells against heat stress. These results indicated that heat-shock may be part of a general stress response, whereas adaptive responses to menadione or hydrogen peroxide are specific and different to that of its. They also suggested that *de novo* protein synthesis is required for adaptive stress responses in yeast cells. Furthermore, Lu, et al. (2005) revealed that at least seven new proteins appear in the *S. cerevisiae* cells following treatment with a sub-lethal dose of hydrogen peroxide (0.2 mM H_2O_2) and potassium chloride (1% KCl), which result in the acquisition of cross-adaptation between hyperosmotic stress and oxidative stress.

Gasch, et al. (2000), analysed the pattern of global gene expression in yeast cells during environmental changes. They found that a large set of genes exhibited similar responses to many different stress conditions. For instance, *CCT1* and *HPS1* genes were responsive to different kinds of stress such as heat-shock and hydrogen peroxide. These genes were induced through the stress response element (STRE) containing promoters, which bind the general stress transcription factors, Msn2p and Msn4p. Induction of gene expression via the stress response element can lead to the protection of yeast cells against many environmental stress conditions (Estruch, 2000). However, some sets of genes showed specific responses to different stress conditions. For example, genes in the *TRX2* cluster were induced by the Yap1p transcription factor in response to hydrogen peroxide, while the expression of those genes that were not dependent on Yap1p were induced in response to heat shock (Gasch, et al., 2000). These results suggest that genes, which respond to environmental changes, could be regulated by different transcription factors depending on the stress conditions, leading to yeast cells adjusting their molecular function to survive in the new environmental stress conditions.

1.3.2 Yeast oxidative stress response

When aerobic organisms are exposed to reactive oxygen species (ROS), oxidative stress will occur when the concentration of these ROS is higher than the antioxidant capacity of the cell. An imbalance between oxidants and antioxidants leads to cell damage (Sies, 1997; Jamieson, 1998 and Morano et al., 2012). ROS are generated by environmental

insults such as exposure to carcinogens, UV and ionizing radiation. In addition to environmental exposure, the normal metabolic processes of aerobic organisms can also produce ROS. A number of researchers revealed that mitochondria are important sites of ROS generation in aerobic organisms including yeast cells. The superoxide anion radical ($O_2^{\cdot-}$) is generated by the leakage of electrons to oxygen via the mitochondria electron transport chain, while the hydrogen peroxide (H_2O_2) is formed by the dismutation of superoxide anions. Also, the hydroxyl radical ($^{\cdot}OH$) is produced via the Fenton reaction, which is the reduction of hydrogen peroxide by iron (Fe^{2+}). In addition, hydroxyl radicals can be generated via the Haber-Weiss reaction and superoxide anion can give electrons to Fe^{3+}, resulting in hydroxyl radical formation (Cadenas and Davies, 2000; Murphy, 2009; Toledano, et al., 2010; Morano, et al., 2012 and Nimse and Pal, 2015). The generation of ROS in yeast cells is shown in Figure 1.1. Therefore, cells have developed antioxidant defences to detoxify ROS and protect cells from oxidative stress. The antioxidant defence systems of *S. cerevisiae* are described in the section below.



Source: Morano, et al. (2012)

Figure 1.1 Reactive oxygen species generation. Superoxide anion ($O_2^{\cdot-}$) can be generated via the electron transport chain in mitochondria. The destruction of superoxide by the enzyme superoxide dismutases leads to the formation of hydrogen peroxide (H_2O_2) in the cell.

1.4 The protective system of *S. cerevisiae* against oxidative stress

Much previous work has classified antioxidant defence systems of the *S. cerevisiae* into two groups, enzymatic defence systems and non-enzymatic defence system (Jamieson, 1998; Herrero, et al., 2008 and Morano, et al., 2012). Enzymatic defence systems consist of enzymes which can act as ROS detoxification and some enzymes act as redox regulators of protein thiols to maintain redox state in yeast cells (Herrero, et al., 2008).

Small molecules such as glutathione (GSH) and thioredoxin, acting as antioxidants are classified as non-enzymatic defence systems (Jamieson, 1998). The antioxidant defence systems will be described in section *1.4.1* and *1.4.2*.

1.4.1 Enzymatic defence systems

It has been reported that there are many enzymes involved in protecting yeast cells against oxidative stress, for example superoxide dismutases, catalase, glutathione reductase, glutathione peroxidase and methionine reductase (Santora and Thiele, 1997; Jamieson, 1998; Estruch, 2000; Herrero, et al., 2008 and Morano, 2012). The functioning of antioxidant defences in the yeast *S. cerevisiae* is summarised in Table 1.2.

There are two catalase enzymes, catalase A, encoded by the *CTA1* gene and catalase T, encoded by *CTT1* gene. These enzymes are located in the peroxisome and cytosol, respectively. The function of catalase A is removing H_2O_2 generated by fatty acid β-oxidation (Jamieson, 1998; Estruch, 2000 and Herrero, et al., 2008). The role of catalase T is less clear, however catalase T is thought to play a more general role as an antioxidant during oxidative stress exposure. The expression of the *CTT1* gene is induced by several varieties of stress including heat, oxidative, osmotic and starvation (Morano, et al., 2012). Furthermore, Grant, et al. (1998) illustrated that deletion of *CTA1* and *CCT1* in *S. cerevisiae* mutants lacking GSH (*gsh1*) or glutathione reductase (*glr1*) exacerbated H_2O_2 sensitivity. This result indicated that catalases may be responsible for protecting the cells against hydrogen peroxide in the absence of glutathione, suggesting that glutathione and catalases provide overlapping defence systems.

Superoxide dismutases (SOD) protect aerobic organisms against oxidative stress by convertion superoxide anion to hydrogen peroxide and oxygen. Two forms of the enzyme superoxide dismutase are found in yeast cells. Firstly there is a copper/zinc-containing SOD (Cu/Zn-SOD), the product of the *SOD1* gene. Cu/Zn-SOD is found in cytoplasm and functions in detoxifying superoxide anions produced by respiration and other means. Yeast *sod1* mutants exhibit growth defects including lysine, methionine and cysteine auxotrophies when grown in aerobic condition and poor growth by respiration (Santora and Thiele, 1997; Jamieson, 1998 and Morano, 2012). Secondly there is a mitochondrial matrix manganese-containing SOD (Mn-SOD), the product of the *SOD2* gene. Mn-SOD is important in protecting the mitochondria from the toxicity

of superoxides generated by respiration (Estruch, 2000). Guidot, et al. (1993) revealed that yeast strains lacking Mn-SOD were unable to grow normally in hyperoxia, an excess of oxygen, whereas a Mn-SOD deficient strain lacking in the mitochondria electron transport chain exhibited normal growth under hyperoxic conditions. These results implied that Mn-SOD plays a role in decreasing hyperoxia mediated stress and also in the detoxification of superoxide anions generated in mitochondria.

Table 1.2 Antioxidant defence systems of yeast

Antioxidant	Gene	Function	Transcription factor
Superoxide dismutases	*SOD1, SOD2*	Dismutation of superoxide anions into H_2O_2 and O_2	Yap1p/Skn7p
Catalases	*CTA1* *CTT1*	Catalyze the reduction of H_2O_2 to H_2O and O_2	Yap1p/Skn7p
Glutathione reductase	*GLR1*	Reduction of oxidised glutathione	
Glutathione peroxidase	*GPX1, GPX2* *GPX3*	Reduction of hydrogen peroxide, Reduction of alkyl hydroperoxides	
Methionine reductase	*MRSA* *MRSB*	Reduction of methionine sulfoxide	
Thioredoxin	*TRX1, TRX2* *TRX3*	Reduction of protein disulphides, Reduction of hydrogen peroxide and alkyl hydroperoxides	Yap1p/Skn7p
Glutathione	*GSH1, GSH2*	Scavenging of free radicals, Conjugation with electrophiles	Yap1p

Source: Adapted from Estruch (2000), Moradas-Ferreira and Costa (2000) and Morano, et al. (2012)

1.4.2 Non-enzymatic defence systems

Thioredoxin is a small protein with two redox-active cysteine residues in an active centre (Cys-Gly-Pro-Cys-) and functions together with NADPH and thioredoxin reductase as an efficient reducing system of exposed protein disulfides (Masutani, et al., 2000). Many studies have reported that the thioredoxin systems in *S.cerevisiae* comprises of cytoplasmic thioredoxin and mitochondrial thioredoxin. The cytoplasmic thioredoxin system contains two thioredoxins, encoded by the *TRX1* and *TRX2* genes, and a thioredoxin reductase, encoded by the *TRR1* gene. The thioredoxin gene, *TRX3*, and the thioredoxin reductase gene, *TRR2* are part of the mitochondrial thioredoxin system (Estruch, 2000; Carmel-Harel and Storz, 2000; Toledano, et al., 2010 and Morano, et al., 2012). Garrido and Grant (2002) indicated that Trx1p and Trx2p have functions as antioxidants. Overexpression of either *TRX1* or *TRX2* resulted in an increase in the resistance of cells towards hydrogen peroxide. While *S. cerevisiae* lacking *TRX2* were hypersensitive to hydrogen peroxide and *tert*-butyl hydroperoxide, whereas the strain containing *TRX2*, in the absence of *TRX1* exhibited wild-type levels of resistance. This result indicated that the *TRX2* seems to play the dominant role in protecting yeast cells against oxidants.

Glutathione (GSH), a tripeptide γ-L-glutamyl-L-cystinylglycine is the most abundant low molecular mass thiol compound found in the cells. GSH functions in cell as an antioxidant and radical scavenger to maintain the redox-balance of the cell. The genes *GSH1*, encoding γ-glutamylcysteine synthetase and *GSH2*, encoding glutathione synthetase are involved in glutathione biosysthesis (Jamieson, 1998; Estruch, 2000; Grant, 2001 and Toledano, et al., 2010). Many studies have been reported that yeast strains lacking *GSH1* are hypersensitive to hydrogen peroxide and superoxide anions (Izawa, et al. 1995; Stephen and Jamieson, 1996 and Grant, et.al. 1998). Whereas the *GSH2* deficient mutant was more resistant to hydrogen peroxide than the *gsh1* mutant (Grant, et al., 1997). Exposure of yeast cells to hydrogen peroxide led to decreased GSH levels and the redox balance of the cell shifted to a more oxidized form (Grant, et al., 1998). Grant, et al. (1997) also reported that overexpression of *GSH1* resulted in increased levels of intracellular GSH, but *GSH2* had no effect on the levels of GSH. These findings implied that *GSH1* may play an important role in response to oxidative stress.

1.5 Transcription factors regulate the oxidative stress response

It has been reported that Yap1p and Skn7p transcription factors are majorly involved in the oxidative response, however Msn2/4p transcription factors are also required for protection against oxidative stress (Moradas-Ferreira and Costa, 2000; Toledano, et al., 2010 and Morano, et al., 2012). Yap1p is one of the members of a leucine-zipper (bZip) AP-1 transcriptional factor family and is an oxidant-responsive transcription factor (Herrero, et al., 2008). Yap1p was found to play a key in the regulation of genes encoding yeast antioxidant defences. Kuge and Jones (1994) showed that *S. cerevisiae* cells lacking the *YAP1* gene became hypersensitive to hydrogen peroxide; however, overexpression of *YAP1* resulted in hyper-resistance to the same stress. In addition, exposure of yeast to hydrogen peroxide could induce the expression of yeast genes including *TRX2* and *SSA1* (encoding an HSP70 isoform). The expression of these genes was dependent on Yap1, while the expression of *GSH1* which encodes γ-glutamylcysteine synthetase, was more strongly induced by superoxide anions than hydrogen peroxide (Stephen, et al., 1995).

Regarding the Skn7p transcription factor, evidence shows that Skn7p is also associated with the oxidative stress response. Yeast strains deleted for *SKN7* displayed sensitivity to oxidants such as hydrogen peroxide, cadmium and menadione. Morgan, et al. (1997) showed that the induction of *TRX2* and *TRR1* expression in response to oxidative stress required Skn7p, suggesting that the Skn7p protein binds directly to the *TRX2* promoter and co-operates with Yap1p to induce the expression of *TRX2*. Furthermore, Skn7p has been found to be involved in peroxide induction of various heat-shock genes (*HSP12*, *HSP26* and *HSP104*). Mutants lacking *hsf1* and *skn7* displayed exacerbated hypersensitity to peroxide, indicating that Skn7p might function as a coordinator of Hsf1p in response to peroxide stress (Toledano, et al., 2010 and Morano, et al., 2012).

Apart from Yap1p and Skn7p, the zinc finger transcription factors, Msn2p and Msn4p have been shown to be responsible for a full oxidative stress response. These transcription factors bind the stress response element (STRE) and are required for the induction of *STRE* containing genes in response to a variety of environmental conditions, including heat-shock and oxidative stress (Estruch, 2000). Morades-Ferreira and Costa (2000) also reported that hydrogen peroxide could induce antioxidant defence genes such as *CTT1* and *SOD1* which are regulated by the Msn2/4p transcription factors. Strain lacking Msn2p and Msn4p were hypersensitive to hydrogen peroxide and showed a defect in the induction of *STRE* containing genes in response to hydrogen

peroxide (Toledano, et al., 2010). These studies indicated that the Msn2/4p transcription factors participate in resistance to oxidative stress.

1.6 The aim of this work

The objective of this research is to study the mechanism of action of chemical antioxidants in protecting eukaryotic cells against reactive oxygen species by using *Saccharomyces cerevisiae* as a model system.

This study attempts to examine the effects of chemical antioxidants on yeast cells, to determine whether BHA acts as an antioxidant directly or alternatively by acting as a weak pro-oxidant and inducing an oxidant adaptive stress response. The nature of any stress response induced by BHA will be examined by measuring the expression of key oxidant inducible genes. Additionally the effect of BHA on yeast mutants deficient in a variety of genes will be analysed. A genetic approach toward understanding BHA toxicity will also be undertaken.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Strains

2.1.1 Yeast Strains

The yeast strains used in this study are listed in Table 2.1 and Table 2.2. *Saccharomyces cerevisiae* strains were grown in YPD medium for non-selective growth or SD medium for selective growth. All strains were incubated with shaking at 30 ^{o}C.

Table 2.1 Strains of *S. cerevisiae* used in this study

Strain	Genotype	Source
S150-2B	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-Δ1, Gal+*	D. Jamieson*
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCRAF
BY4742	*MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	EUROSCRAF
Y06913 (ΔSOD1)	BY4741; *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YJR104c::kan MX4*	EUROSCRAF
Y06605 (ΔSOD2)	BY4741; *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YHR008c::kan MX4*	EUROSCRAF
DJY125 (ΔYAP1)	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-Δ1 yap1 :: TRP1 GAL+* S150-2B transformed with *EcoRI*/*SphI* cut *pyap1::TRP1*	D. Jamieson
W303	*MAT***a** *his 3-11, 15 can 1-100 ade 2-1 leu2-3,112 trp 1 ura 3-52*	D. Jamieson
W303 (ΔMSN2/MSN4)	*MAT***a** *ade2 can1 his3 leu2 trp1 ura3 msn2-Δ3 :: HIS3 msn 4-1::TRP1*	D. Jamieson
Y13305 (ΔUBS1)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBR165w::kanMX4*	EUROSCRAF
Y13306 (ΔTYR1)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBR166c::kanMX4*	EUROSCRAF
Y17166 (ΔPEX32)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBR168w::kanMX4*	EUROSCRAF

* D. Jamieson, School of Life Sciences, Heriot-Watt University, Edinburgh, UK

Table 2.1 Strains of *S*. *cerevisiae* used in this study (continue)

Strain	Genotype	Source
Y17167 (ΔSSE2)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YBR169c::kanMX4*	EUROSCRAF
Y16519 (ΔNGL3)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YML118w::kanMX4*	EUROSCRAF
Y16520 (ΔYML119w)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YML119w::kanMX4*	EUROSCRAF
Y16521 (ΔNDI1)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YML120c::kanMX4*	EUROSCRAF
Y16522 (ΔGTR1)	BY4742; *MAT***α** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YML121w::kanMX4*	EUROSCRAF
Y05574 (ΔKRE6)	BY4741; *MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YPR159w::kanMX4*	EUROSCRAF
Y05575 (ΔGPH1)	BY4741; *MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YPR160w::kanMX4* (*gph1::kanMX4*)	EUROSCRAF
Y00257 (ΔPMP2)	BY4741; *MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YEL017c-A::kanMX4* (*pmp2::kanMX4*)	EUROSCRAF
Y00258 (ΔGTT3)	BY4741; *MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YEL017w::kanMX4* (*gtt3::kanMX4*)	EUROSCRAF
Y00259 (ΔEAF5)	BY4741; *MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YEL018w::kanMX4* (*eaf5::kanMX4*)	EUROSCRAF
Y00261 (ΔYEL020c)	BY4741; *MAT***a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YEL020c::kanMX4*	EUROSCRAF

Table 2.2 BHA sensitive yeast mutants used in this study

Strain	Genotype	Source
MS-2	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-3	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-4	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-10	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-12	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-15	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-17	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-18	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-24	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-27	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-28	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-31	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-39	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-41	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-50	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-52	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MS-54	*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1, Gal+*	This study
MB1-2	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-3	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-11	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-15	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-16	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-19	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-21	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-31	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study
MB1-34	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	This study

Table 2.2 BHA sensitive mutants used in this study (continue)

Strain	Genotype	Source
MB2-4	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-17	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-22	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-36	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-38	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-42	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-43	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-44	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-52	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-59	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-64	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-66	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-72	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-75	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-79	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study
MB2-84	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	This study

2.1.2 Bacteria strain

Escherichia coli XL1-blue (*recA1, endA1, gyrA96, relA1, supE44, thi*-1, Δ(lac-proAB), hsdR17(rk-, mk+), [F' proAB, laclqZΔM15::Tn*10*]) was used for amplify and maintain a plasmid. This strain was incubated at 37 ºC.

2.2 Plasmids

Plasmids used in this research are described in Table 2.3 and plasmids map are shown in Figure 2.1-2.3.

Table 2.3 List of plasmids used in this study

Plasmid	Description	Source
pYDJ73 (*GSH1-lacZ*)	*URA3*	D. Jamieson*
pYTRX2-lacZ (*TRX2-lacZ*)	*CEN6/ARS4 URA3TRX2-lacZ*	D. Jamieson
pZJH (*SSA1-lacZ*)	2 micron *URA3 SSA1-lacZ*	D. Jamieson
YEp 13	2 micron, *LEU2*, Amp^R	D. Jamieson
YEp24	2 micron, *URA3*, Amp^R	D. Jamieson
pRS415	*CEN6/ARS H4, LEU2*, Amp^R	D. Jamieson
pYCC1	2 micron, *LEU2*, Amp^R, *ubs1*, *tyr1*, *pop7*, *pex32*, *sse*	This study
pYCC2	2 micron, *LEU2*, Amp^R	This study
pYCC3	2 micron, *LEU2*, Amp^R	This study
pYCC4	2 micron, *LEU2*, Amp^R	This study
pYCC5	2 micron, *URA3*, Amp^R, *kre6*, *gph1*	This study
pYCC6	2 micron, *LEU2*, Amp^R, *ngl3*, *yml119w*, *ndi1*, *gtr1*	This study
pYQZ	2 micron, *URA3*, Amp^R, *pmp2*, *gtt3*, *eaf5*, *yel020c*	This study

* D. Jamieson, School of Life Sciences, Heriot-Watt University, Edinburgh, UK

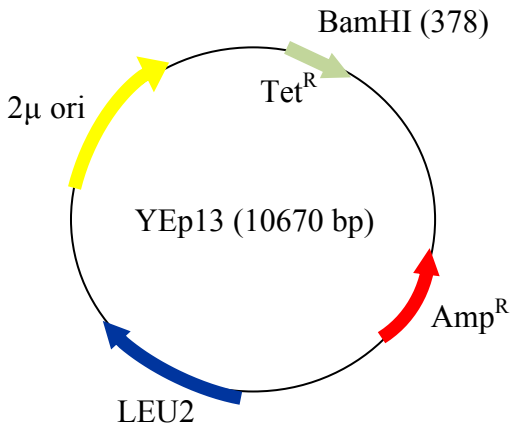


Figure 2.1 Plasmid map of YEp13

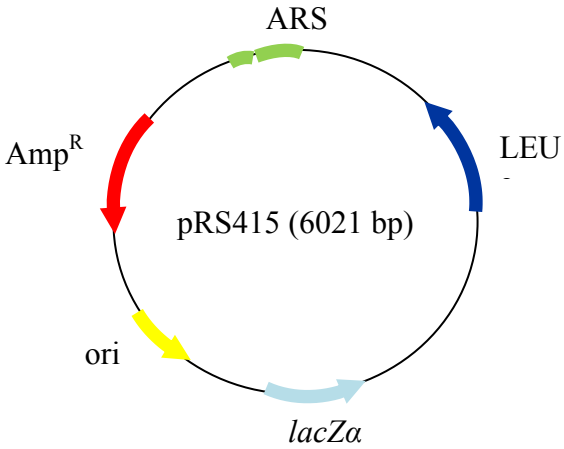


Figure 2.2 Plasmid map of pRS415

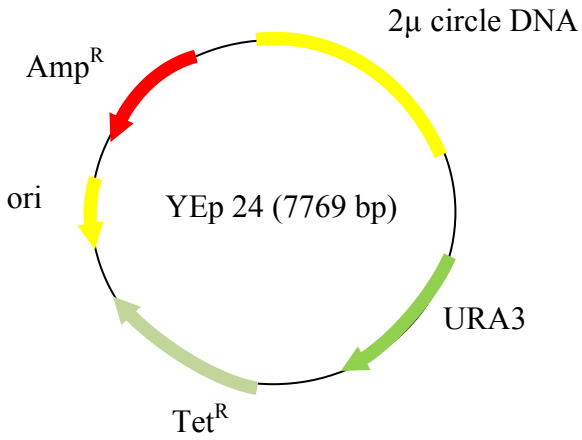


Figure 2.3 Plasmid map of YEp24

2.3 Chemicals, reagents, enzymes and other materials

Chemicals, reagents, enzymes and other materials used in this study were obtained from Fisher Scientific UK, Helena Biosciences, Sigma-Aldrich and Fermentas Life sciences. Chemical stock solutions were autoclaved at 121 ºC for 15 minutes and stored at room temperature or the temperature mentioned in the protocol. In the case of therrmosensitive solutions, sterilization was carried out by using membrane filtration, pore size 0.22 micron.

2.4 Media and Growth conditions

Media were purchased from Difco, Formedium Ltd hunstanton, England or Oxoid Ltd. All media were sterilized by autoclaving at 121 ºC for 15 minutes.

2.4.1 Media and growth condition for S. cerevisiae

YPD medium is a complex media and used for routine yeast growth. SD medium was used for plasmid selection and maintenance. All *S. cerevisiae* strains were incubated at 30 ºC for 2-4 days. Aeration was provided by shaking at 200 rpm when yeast was grown in liquid medium. The components of these media are shown in Table 2.4.

Table 2.4 List of complex medium used in this study

Medium	Component	Composition
YPD	Yeast extract	10 g
	Bacto peptone	20 g
	Glucose	20 g
	Distilled water	1000 ml
Synthetic Dextrose (SD)	Bacto yeast nitrogen base (w/o ammonium sulphate)	1.7 g
	Ammonium sulphate	5 g
	Glucose	20 g
	Amino acid supplement*	20 mg
	Distilled water	1000 ml

* Amino acid supplement was prepared as a stock solution of 10 mg/ml per amino acid. One hundred milligrams of amino acid were added into 10 ml of distilled water. A small pellet of NaOH was added to stock solution in order to help amino acid to dissolve in distilled water. Stock solution of amino acid supplement was sterilized by using

membrane filtration. Addition of amino acid supplement into SD medium depended on yeast strains and plasmids of the study.

Agar 20 g/l was added when solid medium was required.

2.4.2 Media and growth condition for E. coli

LB medium was used for *E.coli* propagation. They were grown overnight at 37 ºC on LB agar, and were also shaken at 250 rpm in LB liquid medium. Components of LB medium are described in table below:

Table 2.5 Components of LB medium

Medium	Component	Composition
LB	Yeast extract	5 g
	Tryptone	10 g
	NaCl	10 g
	Distilled water	1000 ml

For selective medium, ampicillin was added to LB after it had cooled to 45 ºC. Appropriate amounts of an ampicillin stock solution 100 mg/ml (filter-sterilized) was added to LB medium to give a final concentration 50 µg/ml. The solid medium was prepared by adding agar 20 g/l.

2.5 Determining sensitivity towards oxidants and antioxidants

Several methods were used to determine the sensitivity of yeast towards a variety of oxidants and antioxidants

To determine the toxicity of various oxidants and antioxidants towards yeast cells, a paper-disc plate method was used. One hundred microliters of log phase cultures of *S. cerevisiae* S150-2B, obtaining initial cells 10^7 cells/ml approximately were plated out onto YPD agar. After the plate had dried, 10 µl of ethanol 1.88 % (as a solvent only control) and two concentrations (0.1 and 1.0 M) of either BHA, indole-3-carbinol or flavones were spotted on paper discs, which were placed on the surface of these YPD plates previously inoculated with yeast. Inhibition zone diameters were measured after incubation at 30 $^{\circ}$C for 48 hours.

2.5.1 Spot test

To confirm BHA sensitivity of strains, a single colony of each mutant was resuspended into 300 µl sterile water to obtain approximate initial cells 10^7 cells/ml. Serial dilutions (10^0-10^{-4}) are then prepared and 2 µl of each spotted onto YPD or SD plates and plus or minus the oxidant or antioxidant, and then incubated at 30 °C for 3-4 days.

2.5.2 Survival curves and adaptation experiments

To more accurately determine the degree of tolerance cells display toward BHA and H_2O_2, yeast cells were grown aerobically in YPD broth at 30 °C for 12-16 hours to give $O.D_{600} \approx 0.1$-0.2, initial cell numbers were 10^7 cells/ml approximately. One millilitre aliquots of exponential cultures were challenged with increasing concentrations of BHA or H_2O_2. Cultures were then incubated with shaking at 30 °C for 1 hour. For adaptation, log phase cells were treated with a sub-lethal dose of antioxidant for 1 hour and incubated with shaking at 30 °C. Pre-treated cells were then washed twice in fresh YPD medium and then exposed to several concentrations of BHA or H_2O_2 for 1 hour. After that cells were centrifuged at 13,000 rpm for 5 minutes and washed once in 1 ml of sterile distilled water. Appropriate dilutions of cell suspensions were plated onto YPD agar and incubated at 30 °C for 2-3 days. Cell survival was measured by counting colonies on YPD plates.

2.6 β-galactosidase assays

Yeast strains carrying *lacZ* gene fusions to various genes were used to measure gene expression.

Yeast transformants containing a reporter gene plasmid were cultured in SD broth without uracil at 30 °C for 14-18 hours until cells were in early log phase ($O.D_{600} \approx 0.1$-0.3), approximate initial cells 10^7 cells/ml. The non pre-treated or pre-treated (1 hour with 0.05, 0.1, 0.2 and 0.4 mM BHA) cultures were exposed to 0.49 mM H_2O_2 and various concentrations of BHA for 1 hour and also incubated with shaking at 30 °C. Cells were pelleted by centrifugation and resuspended in 1 ml of Z-buffer (pH 7.0). Then cells were permeabilized with 100 µl chloroform and 50 µl of 0.1 % SDS. Cell suspensions were incubated at 28 °C in a water-bath for 5 minutes after that 200 µl of the substrate o-nitrophenyl-β-D-galactoside (ONPG) (4 mg/ml in Z-buffer) was added. After sufficient yellow colour had developed, the reactions were stopped by adding 0.5 ml of 1 M Na_2CO_3. Reactions were centrifuged for 2 minutes at 13,000 rpm and the

A_{420} of the supernatant determined with the units of β-galactosidase activity calculated as described by Steffen (2002). Calculation of β-galactosidase activity present as follows:

$$\beta\text{-galactosidase activity (units)} = 1000 \times \left(\frac{A_{420}}{A_{600} \times \text{volume assayed} \times \text{time (mins)}} \right)$$

Where the volume assayed is the amount of cultured used.

Time is the length of time the reaction was allowed to develop colour in minutes.

A_{600} is the reading obtained from the culture prior to spinning down the cells.

The enzyme activity of each treatment was measured as duplicates of three independent experiments. One-way ANOVA analysis (IBM SPSS Statistics 21) was used to analyse the significance of β-galactosidase assay and results were considered significantly different at $p < 0.05$.

Z-buffer:	$Na_2HPO_4.7H_2O$	8.05g
	$Na_2HPO_4.H_2O$	2.75 g
	KCl	0.375 g
	$MgSO_4.7H_2O$	0.123 g
	Distilled water	500 ml
	pH solution with NaOH to pH 7.0	

2.7 Transformations

2.7.1 E. coli transformation

E. coli transformation was performed when plasmid amplification was required. *E. coli* strains were grown aerobically overnight in LB broth at 37 °C. One millilitre of the overnight culture was transferred to 100 ml of pre-warmed LB media. The culture was incubated with shaking at 37 °C for 1.5-2 hours to give the OD_{600} between 0.2 and 0.4. Then culture was placed on ice for 15 minutes and transferred into Falcon tubes. Cells were spun down in bench centrifuge for 10 minutes at 3,500-4,000 rpm, 4 °C. Cells were

then resuspended in 75 mM $CaCl_2$ and incubated on ice for 20 minutes. Cells were pelleted by centrifugation (4,000 rpm, 10 minutes, 4 °C), resuspended in 4 ml of TFB2 and kept them on ice for 20 minutes prior to use. Five hundred microliter aliquots of competent cells were transferred to eppendorf tubes and stored at -70 °C.

For transformation, frozen competent cells were thawed at room temperature and then placed on ice. Ten microliters of DNA were added to 50 µl of TMC in eppendorf tube. Competent cells, 100 µl, were added to the DNA solution and mixed by inversion. They were then incubated on ice for 30 minute followed by incubation at 42 °C for 5 minutes. After that, 1 ml of LB liquid medium was added and incubated for 30 minutes to 1 hour at 37 °C. Cells were centrifuged at 13,000 rpm for 30 second and resuspened in residual media. One hundred microliters of cells suspension were plated out onto LB-ampicillin agar plates and incubated overnight at 37 °C.

TFB2:

Component	Volume (ml)
10 mM MOPS* pH7.0	10 ml of 100 mM MOPS pH7.0
75 mM $CaCl_2$	7.5 ml of 1M $CaCl_2$
10 mM RbCl	1 ml of 1M RbCl
15% glycerol	15 ml glycerol
Distilled water	66.5 ml
Total volume	100 ml

* MOPS solution was sterilized by membrane filtration

TMC:

Component	Volume
10 mM Tris/HCl pH 8.0	100 µl of 1M Tris/HCl pH 8.0
10 mM $MgCl_2$	100 µl of 1M $MgCl_2$
10 mM $CaCl_2$	100 µl of 1M $CaCl_2$
Distilled water	9.7 ml
Total volume	10 ml

2.7.2 Yeast transformation

Yeast transformation was carried out by the lithium acetate method (Gietz and Woods, 2002). Yeast strains were inoculated into 5 ml of double strength YPD broth (2x YPD) and incubated overnight at 30 ºC with shaking 200 rpm. The next day, the yeast overnight culture was transferred to 50 ml of 2x YPD liquid and the culture was incubated with shaking at 30 ºC for 3-5 hours to give cell titer of at least $2x10^7$ cells/ml. Cells were harvested by centrifugation at 3,000 g for 5 minutes. Cells were then washed in 25 ml of sterile water and resuspended in 1 ml of sterile water. Cell suspensions were transferred to a 1.5 ml microcentrifuge tube, centrifuged for 30 seconds at 13,000 rpm and the supernatant was removed. Water was added to make up final volume of 1 ml. Cells were resuspended by vortex mixing. One hundred microliters of cell suspension was pipetted into 1.5 ml microcentrifuge tubes, centrifuged at top speed for 30 second after which the supernatant was removed. Transformation mix, 360 µl, was then added to the cell pellet of each transformation tube and vortex mixed vigorously. The tube was then incubated in a water bath at 42 ºC for 40 minutes. Then the cells were spun down at top speed for 30 seconds and the tranformation mix was removed using a micropipettor. Sterile water 1 ml was pipetted into the tube and cells were resuspended by stirring with a micropipette tip and vortexed gently. One hundred microliters of the resuspended cells were plated out onto appropriate SD selective medium, incubated at 30 ºC for 3-4 days and transformants were isolated by picking and colony purification.

Transformation Mix:	Component	Volume (µl)
	50% v/v PEG solution	240 µl
	1 M LiAc solution	36 µl
	2 mg/ml Carrier DNA	50 µl
	Plasmid DNA in H_2O	36 µl
	Total volume	360 µl

2.8 Mutagenesis and mutant isolation

2.8.1 Mutagenesis

Yeast cells were grown aerobically in 25 ml YPD broth at 30 °C for 2 days. Stationary phase cultures, 1.5 ml, were transferred to two eppendorf tubes and centrifuged at top speed in the bench centrifuge. Cells were resuspended in 1 ml of 0.1 M sodium phosphate buffer pH 7.0, this wash step was repeated three times. Thirty microliters of ethyl methanesulfonate (EMS) solution were added to one of the tubes, whereas the other tube was used as a no EMS (control). These tubes were then incubated with shaking at 30 °C for 1 hour. Cell suspensions were pelleted and resuspended in 1 ml of 0.1 M sodium phosphate buffer. Then cells were centrifuged and resuspended in 1 ml of 5% sodium thiosulphate (to inactivate the EMS) after that cells were washed in 1 ml of 0.1 M sodium phosphate buffer. Finally, the cell pellet was resuspended in 1 ml of sodium phosphate buffer and stored at 4 °C till used. To determine the degree of mutagenesis, a viable cell count of the mutagenized and nonmutagenized tubes was performed by spread plating serial dilutions onto YPD agar plates. Colonies were counted after incubation at 30 °C for 2-3 days.

2.8.2 Mutant isolation

To isolate BHA-sensitive mutants, replica plates were carried out. One hundred microliters of mutagenized cells were transferred into 10 ml YPD broth, and then incubated with shaking at 30 °C for 2 days. Appropriate dilutions of these mutagenized cells were prepared to give between 20 to 200 viable cells per plate on YPD agar after incubation at 30 °C for 2-3 days. These plates were replica plated to fresh YPD agar, YPD agar containing 0.5 mM BHA and YPD agar containing 0.8 mM BHA. The plates were incubated at 30 °C for 2-3 days. Any colonies that could not grow on YPD containing 0.5 mM BHA or YPD containing 0.8 mM BHA or both plates were candidates for BHA-sensitive mutants.

2.9 Complementation test

Complementation tests were carried out in order to determine whether two mutants are mutated in the same or different genes. Two different mating type mutant strains were crossed on YPD agar plates and then incubated overnight at 30 °C. The resulting diploid yeast from two mutants was isolated on appropriate selective media (SD agar containing

histidine, leucine and uracil). The phenotype of the diploids was scored by spot tests on YPD agar containing 0.5 and 0.8 mM BHA.

2.10 Isolation of plasmid DNA from yeast

A single colony of a yeast transformant was grown aerobically in liquid SD selective medium at 30 °C for 16-18 hours. Ten millilitres of culture was spun down and resuspended in residual medium. Cells were resuspended into 250 µl of resuspension solution. Glass beads, 200 µl were added to the suspension and then vortexed at maximum speed for 3-5 minutes. After that, plasmids were purified by using a GeneJet™ plasmid miniprep kit. Yeast plasmids were amplified in *Escherichia coli* by transformation as described in section *2.7.1.* and purified using a GeneJet™ plasmid miniprep kit.

2.11 Preparation of plasmid DNA from *E. coli*

Plasmid DNA from *E. coli* transformants was prepared by GeneJet™ plasmid miniprep kit Fermentas. A single colony of *E. coli* transformant containing desired plasmid was inoculated into 10 ml of LB-amp liquid medium. The culture was propagated overnight at 37 ºC with shaking. Five millilitres of the overnight culture were harvested by centrifugation at 8,000 rpm for 10 minutes. Supernatant was removed and pelleted cells were used for plasmid DNA by following a GeneJet™ miniprep kit instructions. DNA was eluted in 50 µl of elution buffer and stored at 4 ºC.

2.12 Agarose gel electrophoresis

Plasmid was analysed by agarose gel electrophoresis. Purified plasmid DNA was transferred to an eppendorf tube and digested by appropriate restriction enzymes. Components of the reaction were added in the order listed below:

Component	Volume (µl)
Purified plasmid DNA (1µg approximately)	1 µl
Restriction enzyme buffer	2 µl
Sterile water	16 µl
Restriction enzyme (10U/ µl)	1 µl
Total volume	20 µl

All components were gently mixed and incubated at 37 ºC for 1 hour (temperature and time for incubation depended on the enzyme). Digested plasmid DNA was loaded into a 0.8 % w/v agarose gel and analysed by electrophoresis. Agarose gels were prepared by adding agarose to 30 ml of 1x TAE buffer to make a 0.8% w/v solution and heated in microwave until the agarose was completely melted. When the agarose solution had cooled down to 55ºC, ethidium bromide (10 mg/ml) 1 µl was added to the agarose solution. The agarose was then poured into a gel tray. Once the agarose had become solid, the well comb was pulled out and 1x TAE buffer was poured over the gel. Two microliters of loading dye buffer was added to each of digested plasmids and then 20 µl of each sample was loaded into the well of the gel by using micropipettor. DNA ladder (1 kb) which purchased from Fermentas, 5 µl was pipetted into the first lane of the gel. The voltage of electrophoresis and running time were set at 45-55 Volts and 50 minutes, respectively.

50x stock TAE buffer:	Component	Volume
	Tris-base	242 g
	100% acetic acid	57.1 ml
	0.5 M sodium EDTA	100 ml
	Made up volume to 1,000 ml with distilled water	

1x TAE buffer:	Component	Volume
	50x TAE buffer	20 ml
	Distilled water	980 ml

2.13 Sequencing of genomic DNA inserts

To identify genes responsible for complementing the BHA sensitivity of the mutants, the primers are complementary to plasmid sequences adjacent to the cloning site used in this study.

Name	Sequence	Source
ox164	5'-TCACTATGGCGTGCTGCTAGCGCT-3'	Eurofins Genomics
ox165	5'-CTGCCACCATACCCACGCCGAAAC-3'	Eurofins Genomics
ox517	5'-TGCGTTGATGCAATTTCTATG-3'	Eurofins Genomics

2.14 Measuring growth of yeast strains

To examine growth yeast, cell density was measured by spectrophotometer by measuring the optical density (OD) at 600 nm.

CHAPTER 3 - EFFECT OF ANTIOXIDANTS ON YEAST

An antioxidant is defined as any substance (natural or synthetic), which when present at low levels can delay or inhibit oxidation of key biomolecules (Sies, 1997). It has been reported that antioxidants play a key role in protecting cells against oxidative stress, which is an imbalance between oxidants (e.g. HO^-, H_2O_2 and O_2^-) and antioxidant buffering capacity, leading to cellular damage (Sies, 1997; Jamieson, 1998). Some chemical antioxidants, for example; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and butyl hydroquinone (TBHQ) are widely used in many products. For example, BHA and BHT have been used in food products, animal feed, cosmetics and petroleum products (IARC, 1986; Williams, et al., 1999; Anonymous, 2011). These antioxidants can act as free radical terminators, oxygen scavengers and chelating agents. Antioxidants such as thiodiopropionic acid and dilauryl theodipropionate are able to break down the hydrogen peroxide formed during lipid oxidation into stable end products (Sen and Chakraborty, 2011). Additionally, BHA was found to show protective ability by inducing phase II detoxifying enzymes like glutathione S-transferase, quinine reductases and UDP-glucoronosyl transferases. These enzymes can catalyze the metabolic detoxification of carcinogens and protect cells from oxidative stress and there is also some indication that they may function by inducing oxidant adaptive stress responses (Yu, et al., 2000).

As described above, oxidative stress is a harmful condition caused by the excess of reactive oxygen species (ROS) and/or a decrease in antioxidant levels, resulting in the damage of cell structures. Generally, ROS refers to superoxide anion ($O_2^{\cdot-}$), hydroxyl (OH), peroxyl ($ROO^{\cdot}$) and hydrogen peroxide (H_2O_2). They are generated naturally during normal aerobic cell metabolism (Sen and Chakraborty, 2011). Much previous research about yeast oxidative response had used external oxidants such as hydrogen peroxide (H_2O_2) and menadione (a superoxide generating agent). This work demonstrated that pretreatment of yeast with low levels of oxidants (H_2O_2 or O_2^-) could lead to cellular adaptation to toxic levels of these oxidants (Jamieson, 1992; Jamieson, et al., 1996; Biryukova, 2008). Izawa, et al. (1995) indicated that increases in the levels of intracellular glutathione plays a key role in adaptive response towards H_2O_2 stress. The aim of the work outlined in this chapter is to examine the effect of antioxidants on the yeast oxidative adaptive stress response to test the hypothesis that some antioxidants function by acting as weak pro-oxidants. The effects of antioxidants on gene expression will be discussed in chapter 4.

3.1 Sensitivity of yeast strains to a variety of antioxidants

Three chemical antioxidants (BHA, indole-3 carbinol and flavone) which are widely used in many products were examined in this study. In order to determine the ranges of antioxidant concentrations that can be tolerated by yeast cells, the sensitivity of *S. cerevisiae* towards three antioxidants (BHA, indole-3-carbinol and flavone) was examined. In initial experiments, zones of inhibition were analysed by a paper-disc plate method and these experiments suggested that these compounds were in fact toxic to yeast. Log phase cultures of *S. cerevisiae* S150-2B were plated out onto YPD agar and ten microliters of 96 %ethanol (control) and two concentrations (0.1 and 1.0 M) of either BHA, indole-3-carbinol or flavone were spotted onto paper discs which were laid on YPD plates. Inhibition zone diameters were measured after incubation at 30 $^{\circ}$C for 48 hours. The results revealed that at high levels of BHA (1.0 M) were toxic (diameter of clear zone 22 mm), there was even some toxicity at lower levels (Figure 3.1). Whereas inhibition zones of indole-3-carbinol and flavone were lower than BHA at the same concentration (Table 3.1). It appeared that BHA was more toxic to S150-2B than either indole-3-carbinol or flavone.

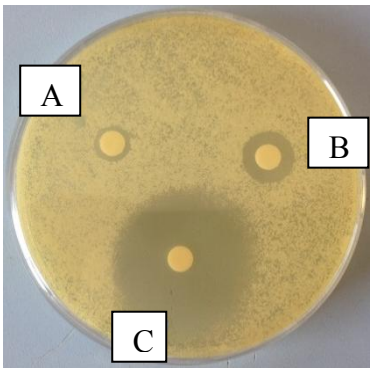


Figure 3.1 Inhibition zone of *S. cerevisiae* S150-2B against BHA 0.1 M (B) and 1.0 M (C), (A) solvent only control (10 µl - 96% ethanol).

Table 3.1 Inhibition zone of *S. cerevisiae* strain S150-2B against different concentrations of antioxidants

Antioxidants	Diameter of clear zone (mm)*		
	96% Ethanol	0.1 M	1.0 M
BHA	8±1	11±1	22±2
Indole-3-carbinol	6±0	10±1	15±1
Flavone	8±1	12±2	14±2

* Diameter of paper-disc is 6 mm. Data is shown is average of three independent experiments.

To confirm the results of the plate assays, quantitative sensitivity experiments were carried out by treating exponential broth cultures of S150-2B with various concentrations of either BHA, indole-3-carbinol or flavone for 1 hour. Cell viability was then examined by plating out onto YPD agar at appropriate dilutions. Numbers of viable cells (colonies) were counted after incubation at 30 $^{\circ}$C for 48 hours. The results demonstrated that BHA was more toxic to yeast cells compared to indole-3-carbinol and flavone and correlated well with the results of the plate tests (Figure 3.2). For example there were no surviving cells after the S150-2B cultures were exposed to 1 mM BHA and 10 mM indole-3-carbinol. The survival of flavone-treated cells decreased to zero only after challenge with the highest concentration (80 mM flavone). These results implied that S150-2B cells were sensitive to BHA more than indole-3carbinol and flavone. Conceivably BHA could induce oxidative damage including DNA damage and there are some reports that suggest that that may be the case (Ali and Suzuki, 2012; Williams, et al., 1999). The toxicity of BHA (a known food additive/ antioxidant) was examined further in order to study how this antioxidant compound acts in yeast cells.

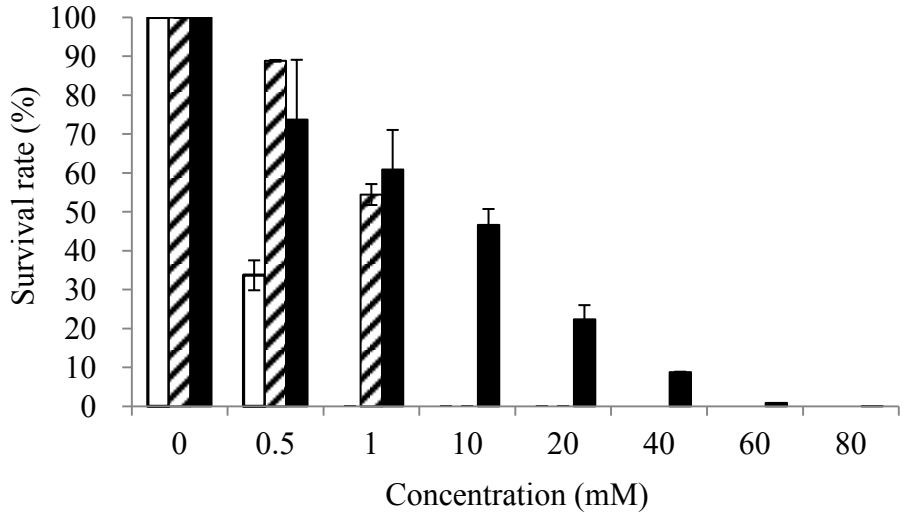


Figure 3.2 Survival of *S. cerevisiae* S150-2B exposed to different kinds of antioxidants (BHA (□), indole-3-carbinol (▨) and flavone (■)). Exponentially growing cells, $O.D_{600} \approx 0.1$-0.2 were treated with various concentrations of antioxidants (0.5, 1, 10, 20, 40, 60 and 80 mM) for 1 hour at 30 °C. Data shown as means ± S.D. (n = 4).

3.2 Pre-exposure to sub-lethal doses of BHA induces protection towards normally toxic levels of BHA

The previous experiments posed several questions: could sub-lethal doses of BHA induce an adaptive stress response and was the toxicity the result of oxidative stress?

It has been demonstrated that when yeast cells are exposed to mild environment stresses such as oxidants (H_2O_2, O_2^- or OH^-) and heat, resulting acquire some level of stress resistance (Jamieson, 1992; Izawa, et al., 1995; Hohmann and Mager, 2010). To determine whether pre-exposure of yeast cells to BHA induced an adaptive stress response, cultures of *S. cerevisiae* were exposed to low levels of BHA and then challenged with normally toxic levels of the same compound, with the degree of protection afforded measured using cell viability. From the data in Figure 3.3, it is apparent that BHA pre-treated *S. cerevisiae* became more resistant to toxic levels of BHA. Especially, at the concentration 0.6 and 0.8 mM BHA, with the survival rate of adapted cells being increased three times that of non-adapted cells. The results of these experiments show that *S. cerevisiae* possess an adaptive response to BHA, however, adapted yeasts were still sensitive to the highest levels of BHA.

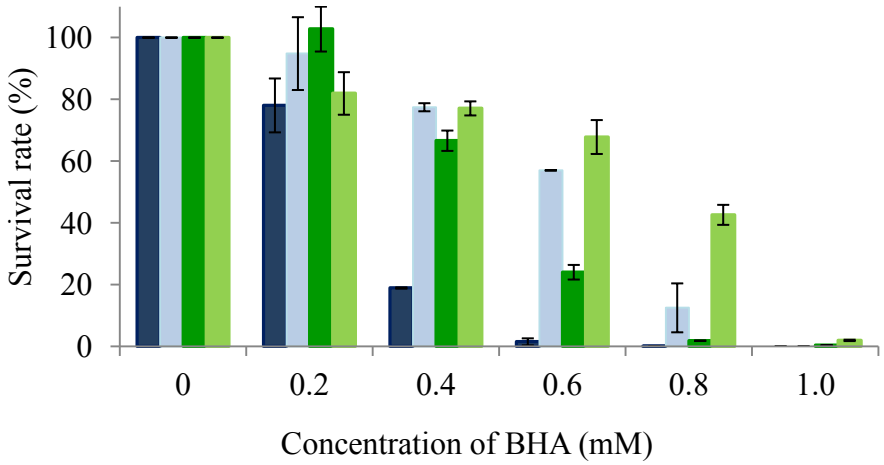


Figure 3.3 Effect of BHA on cell survival of *S. cerevisiae* S150-2B and DJY125 without pre-treatment and pre-treatment with BHA (0.2 mM, 1 hour). Exponential phase cultures of S150-2B (wild type) and DJY125 (Δ*yap1*) were exposed to varying concentration of BHA for 1 hour at 30 ºC. For the adaptation experiments, cells in exponential phase were pre-treated with a sub-lethal dose of BHA (0.2 mM) for 1 hour. Cells were then exposed to increasing concentrations of BHA for 1 hour at 30 ºC. Data shown is the mean ± S.D. (n = 4).

Symbols: non-pretreated S150-2B; pre-treated S150-2B; non-pretreated DJY125 (Δ*yap1*); pre-treatedDYJ125 (Δ*yap1*)

Having determined that pre-exposure towards BHA induces an adaptive response, therefore BHA response was examined further in order to test if this response was identical to the relatively well-characterised oxidative stress responses. As a first step the BHA sensitivity of several strains lacking key proteins involved in countering oxidative stress was determined. These strains were challenged with increasing levels of BHA for 1 hour and cell viability was determined. The results illustrated that the mutants deleted for; the Yap1-transcription factor (DJY125), a key regulator of the oxidative stress response in yeasts (Ouyang et al., 2011), a strain lacking the Msn2/Msn4 -zinc finger transcription factors (W303 (Δ*msn2*/*msn4*)), key regulators of the general stress response (Gasch, 2010), and finally lacking the *SOD1* (Y06913) and *SOD2* (Y06605) genes, which are important in removing superoxide anions (Estruch, 2000), were all slightly decreased in cell viability as the concentration of BHA increased (Figure 3.3- Figure 3.5). Interestingly, as can be seen from Figure 3.3, cell

viability of non pre-treated DJY125 (Δ*yap1*) was higher than the wild type strain (S150-2B). While non-pretreated Δ*sod2* mutants exhibited the same level of sensitivity against BHA as the isogenic wild type strain (BY4741) (Figure 3.4). No significant differences in cell viability were found between non-pretreated W303 cells and the *msn2/4* deletion mutant (Figure 3.5). These findings indicate that BHA toxicity may not directly involve the classical oxidant stress responses in yeast cells.

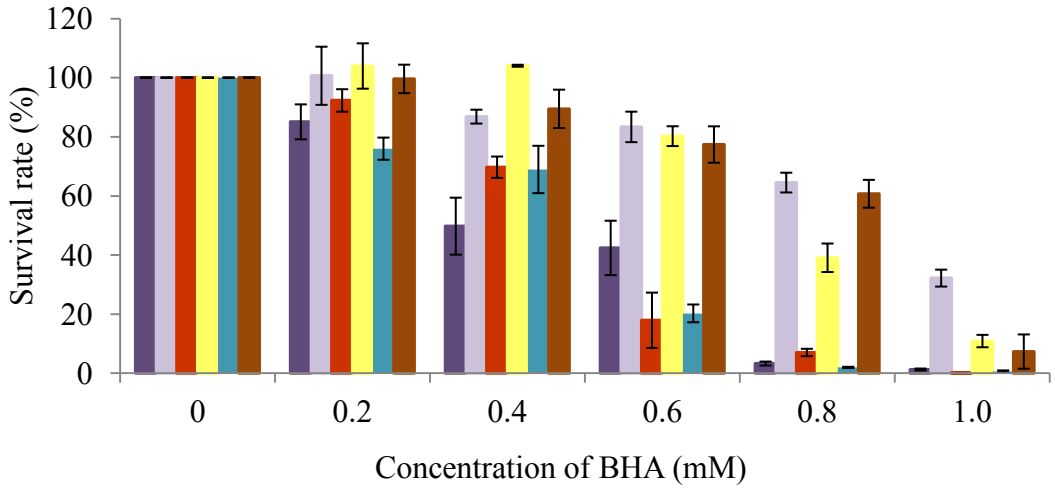


Figure 3.4 Effect of BHA on cell survival of *S. cerevisiae* oxidative stress sensitive mutants with and without pre-treatment and with BHA (0.2 mM, 1 hour). Exponential phase cultures of BY4741 (wild type), Y06913 (Δ*sod1*) and Y06605 (Δ*sod2*) were exposed to increasing concentrations of BHA for 1 hour at 30ºC. For adaptation, cells in exponential phase were pre-treated with sub-lethal dose of BHA (0.2 mM) for 1 hour. Cells were then exposed to increasing concentrations of BHA for 1 hour at 30 ºC. Data is shown as means ± S.D. (n = 4).

Symbols:
- non-pretreated BY4741
- non-pretreated Y06913 (Δ*sod1*)
- non-pretreated Y06605 (Δ*sod2*)
- pre-treated BY4741
- pre-treated Y06913 (Δ*sod1*)
- pre-treated Y06605 (Δ*sod2*)

To determine whether or not a sub-lethal dose of BHA could still induce an adaptive response to BHA in these mutants, survival rate of these mutants (DJY125 (Δ*yap1*), Y06913 (Δ*sod1*), Y06605 (Δ*sod2*) and W303 (Δ*msn2*/*msn4*)) and their corresponding wild type strains (S150-2B, BY4741 and W303) were examined by pre-treatment those yeast strains with 0.2 mM BHA and then exposed to increasing levels of BHA. The

results show that the cells viability of adapted mutants (DJY125 (Δ*yap1*), Y06913 (Δ*sod1*), Y06605 (Δ*sod2*) and W303 (Δ*msn2/msn4*)) was higher than non-adapted cells. The wild type strains (S150-2B, BY4741 and W303) pre-treated with sub-lethal dose of BHA (0.2 mM) also exhibited increasing in resistance to toxic levels of BHA. The survival rate of these adapted wild type cells was around two fold higher than non-adapted cells (Figure 3.3- Figure 3.5). These results show that pre-treatment yeast cells with sub-lethal dose of BHA could still induce an adaptive response to BHA even in strains deficient in oxidant stress resistance.

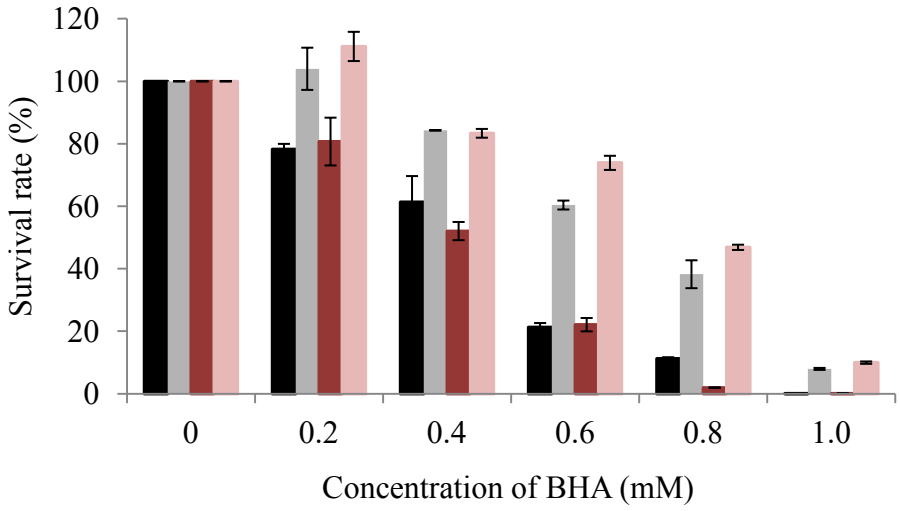


Figure 3.5 Effect of BHA on cell survival of *S. cerevisiae* Msn2/4 deficient strains with and without pre-treatment with BHA (0.2 mM, 1 hour). The exponential phase cultures of W303 (wild type) and W303 (Δ*msn2/msn4*) were exposed to several concentrations of BHA for 1 hour at 30 ºC. For adaptation, cells in exponential phase were pre-treated with sub-lethal dose of BHA (0.2 mM) for 1 hour. Cells were then exposed to increasing concentrations of BHA for 1 hour at 30 ºC. Data is shown as means ± S.D. (n = 4).

Symbols: non-pretreated W303; pre-treated W303; non-pretreated W303 (Δ*msn2/msn4*); pre-treatedW303 (Δ*msn2/msn4*)

3.3 Does BHA pre-treatment affect the sensitivity towards H_2O_2?

As many environmental and chemical stresses have at their root the production of oxidative stress, exposure to one stress frequently induces cross-protection against another (Jamieson, 1992; Jamieson, et al., 1996; Gasch, 2010). Therefore, determination of the sensitivity of BHA-pretreated cells and non-pretreated cells towards H_2O_2 was carried out in order to investigate whether BHA affected the adaptive stress response to H_2O_2.

As shown in Figure 3.6, non-pretreated cells of the strain lacking Yap1 (DJY125) were sensitive to H_2O_2. The cell viability of this mutant was significantly decreased when compared with the wild type cells. This is because the Yap1 transcription factor plays an important role in H_2O_2 tolerance and regulates the expression of genes that respond to H_2O_2 and the thiol-oxidant diamide (Carmel-Harel, et al., 2001; Lee, et al., 1999; Herrero, et al., 2008). In addition, similar results were observed in W303 *msn2*/*msn4* deleted strain (Figure 3.8). Non-pretreated cells of the Δ*msn2*/*msn4* mutant were more sensitive to H_2O_2 than wild type. Conversely, the strains lacking *sod1* and *sod2* displayed similar sensitivity to H_2O_2 to the wild type strain (Figure 3.7). From the data in Figure 3.6- Figure 3.8, it was noticed that the survival of BHA-pretreated cells was approximately 3-4 times higher than non-pretreated cells. These results demonstrated that although non-pretreated cells were sensitive to H_2O_2, cells pretreated with 0.2 mM BHA showed a significant increase in the degree of resistance to H_2O_2. An implication of these findings is that this antioxidant also leads to cross-protection against H_2O_2 by inducing directly or indirectly the H_2O_2 adaptive response.

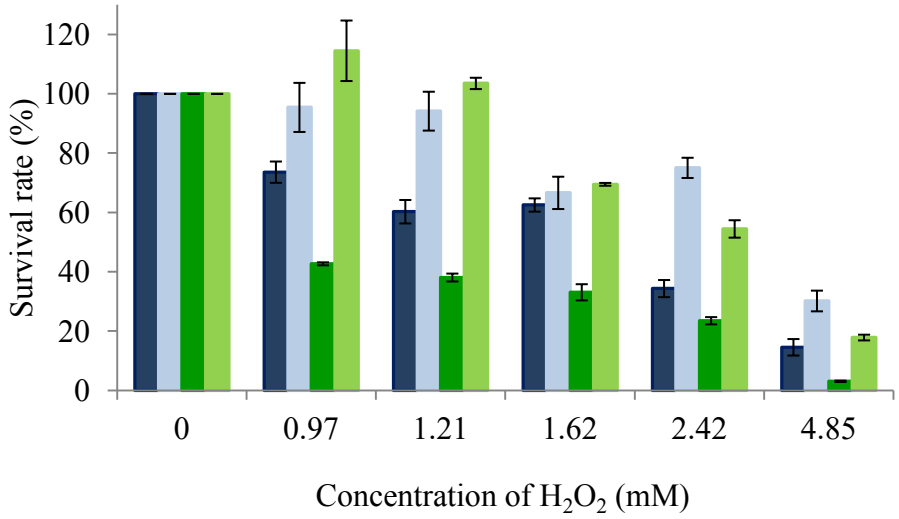


Figure 3.6 Viability of non-adapted and adapted *S. cerevisiae* strains (S150-2B and DJY125) to H_2O_2. Cells of S150-2B (wild type) and DYJ125 (Δ*yap1*) in exponential phase were directly treated with increasing concentrations of H_2O_2 without pre-treatment for 1 hour at 30 ºC. Adapted cells were treated with sub-lethal dose of BHA (0.2 mM, 1 hour) and then exposed to increasing levels of H_2O_2 for 1 hour at 30 ºC. Data is shown are the means ± S.D. (n = 4).

Symbols: non-pretreated S150-2B; pre-treated S150-2B; non-pretreated DJY125 (Δ*yap1*); pre-treatedDYJ125 (Δ*yap1*)

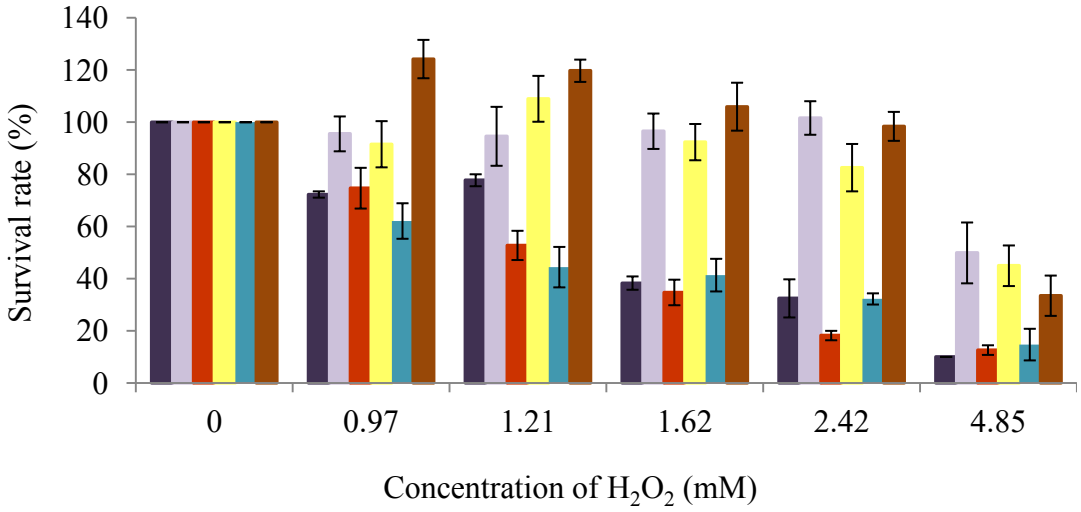


Figure 3.7 Viability of non-adapted and adapted *S. cerevisiae* strains (BY4741, Y06913 and Y06605) to H_2O_2. Cells of BY4741 (wild type), Y06913 (Δ*sod1*) and Y06605 (Δ*sod2*) in exponential phase were directly treated with various concentrations of H_2O_2 without pre-treatment for 1 hour at 30 ºC. Adapted cells were treated with sub-lethal dose of BHA (0.2 mM, 1 hour) and then exposed to increasing levels of H_2O_2 for 1 hour at 30 ºC. Data shown as means ± S.D. (n = 4).

Symbols:
- non-pretreated BY4741
- pre-treated BY4741
- non-pretreated Y06913 (Δ*sod1*)
- pre-treated Y06913 (Δ*sod1*)
- non-pretreated Y06605 (Δ*sod2*)
- pre-treated Y06605 (Δ*sod2*)

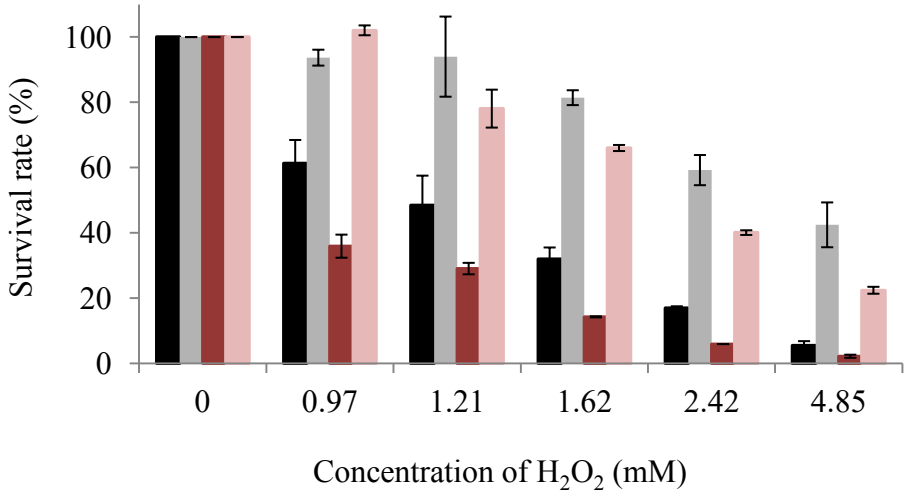


Figure 3.8 Viability of non-adapted and adapted *S. cerevisiae* strains (W303 and W303 (Δ*msn2*/*msn4*)) to H_2O_2. Cells of W303 (wild type) and W303 (Δ*msn2*/*msn4*) in exponential phase were directly treated with various concentrations of H_2O_2 without pre-treatment for 1 hour at 30 ºC. Adapted cells were treated with sub-lethal dose of BHA (0.2 mM, 1 hour) and then exposed to increasing levels of H_2O_2 for 1 hour at 30 ºC. Data shown as means ± S.D. (n = 4).

Symbols: non-pretreated W303 pre-treated W303
non-pretreated W303 (Δ*msn2*/*msn4*) pre-treatedW303 (Δ*msn2*/*msn4*)

CHAPTER4 – THE EFFECT OF BHA ON GENE EXPRESSION

Oxidative stress occurs when there is overproduction of ROS. However, all aerobic cells have antioxidant defences system to protect themselves from oxidative stress. For yeast cells, there are two broad defence systems that play a key role in protecting cells against oxidants. One is a non-enzymatic defence system, consisting of small molecules such as glutathione and thioredoxin which can act as radical scavengers that remove oxidants. Second is a number of cellular enzymes, for example; catalase, superoxide dismutase, glutathione reductase and thioredoxin peroxidise which are able to inactivate a variety of oxidants (Jamieson, 1998). In addition, studies at a molecular level have shown that several yeast genes (e.g., *SOD1*, *SOD2*, *TRX2*, and *GSH1*) are involved in protection against oxidative stress. These yeast genes were found to be induced by oxidants (Stephen et al., 1995; Santoro and Thiele, 1997; Estruch, 2000). The expression of *TRX2* gene (encoding thioredoxin 2) and *GSH1* gene (encodes γ-glutamlycysteine) can be induced by H_2O_2. These genes are also regulated by the Yap1 transcription factor, which responds to oxidative stress in yeast (Stephen, et al., 1995; Grant, 2001; Carmel-Harel, 2001). In this chapter the expression of some oxidant-regulated genes were determined in order to investigate whether or not these oxidant-regulated genes were also induced by BHA.

4.1 Does exposure towards BHA induce expression of oxidant-regulated genes?

Having shown that sub-lethal levels of BHA could induce a protective response to toxic levels of both BHA and H_2O_2, therefore the experiment was examined further to see if BHA exposure induced the expression of some key known oxidant inducible genes. The expression of a number of genes in response towards various form of oxidative stress has been studied in some detail, in particular the *GSH1*, *TRX2* and *SSA1* genes; encoding γ-glutamylcysteine synthetase which is involved in with glutathione biosynthesis, thioredoxin 2 and Ssa1 a stress inducible HSP70 isoform respectively (Santoro and Thiele, 1997). Expression of these genes in response to BHA exposure was conveniently measured using yeast strains carrying *lacZ* gene fusions to these genes. Yeast strain S150-2B was transformed by the plasmids carrying the gene fusions and the resulting transformants exposed to a variety of concentrations of H_2O_2 and BHA and the resulting activity of β-galactosidase measured as described in chapter 2 section 2.6.

The changes in gene expression of *TRX2-lacZ*, *GSH1-lacZ* and *SSA1-lacZ* are shown in Figure 4.1- Figure 4.3. From these data show that expression of the three yeast genes (*TRX2, GSH1* and *SSA1*) was increased by H_2O_2. This result was consistent with previous results, demonstrating that oxidants such as hydrogen peroxide and superoxide anion could induce the expression of the *TRX2*, *SSA1* and *GSH1* genes (Stephen, et al., 1995).

In non-adapted S150-2B treated with several different concentrations of BHA, the expression of the *TRX2* and *GSH1* genes was not significantly different from the levels of expression found in non-BHA treated cells ($p > 0.05$) (Figure 4.1 and Figure 4.3). The expression levels of the *TRX2-lacZ*, *SSA1-lacZ* and *GSH1-lacZ* gene fusions in cells exposed to between 0.6-1.0 mM BHA were lower than that non-BHA treated cells, this may be an artefact due BHA induced cell death (Figure 3.3).

Interestingly, there was a significant difference in expression of the *SSA1-lacZ* gene fusion between the control and cells challenged with 0.4 mM BHA ($p < 0.05$) (Figure 4.2). It seems heat shock gene *SSA1* probably participate in BHA stress response. Additionally, cells pre-treated with low levels of BHA (0.05, 0.1, 0.2 and 0.4 mM BHA) and then subsequently exposed to 0.49 mM H_2O_2 exhibited lower levels of expression of the *TRX2-lacZ* and *SSA1-lacZ* gene fusions compared to those cells treated with H_2O_2 alone (Figure 4.1- Figure 4.2). Whereas expression of the *GSH1-lacZ* gene fusion in BHA pre-treated cells was slightly higher than that found in H_2O_2-treated cells (Figure 4.3), suggesting that expression of *TRX2*, *SSA1* and *GSH1* were more strongly induced by H_2O_2 than BHA. It seems BHA could not induce the expression of these genes. These observations are consistent with BHA acting as an antioxidant and also confirm that pre-treatment yeast cell with low levels of BHA may not induce gene expressions which respond to oxidative stress.

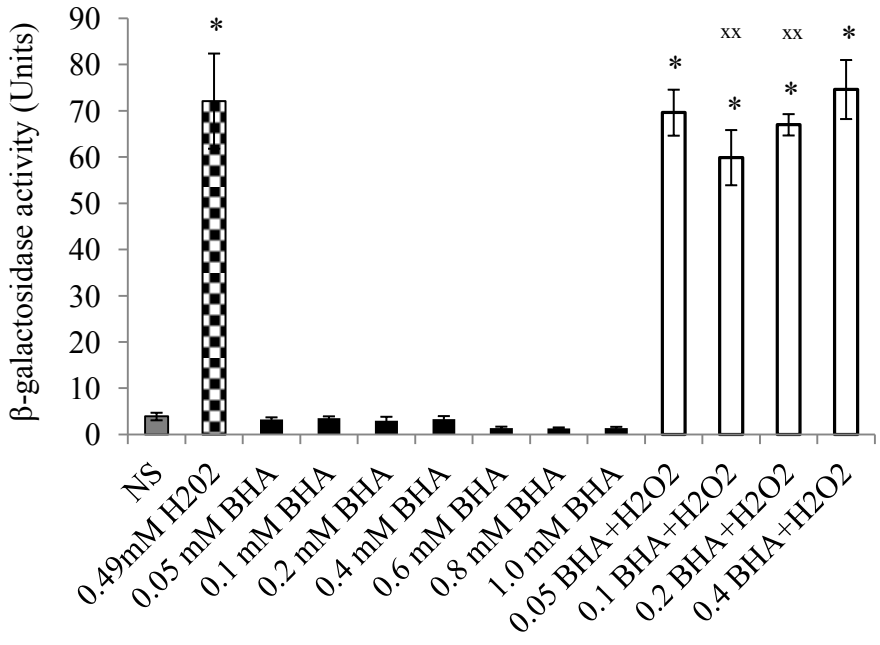


Figure 4.1 The effect of BHA on the induction of *TRX2-lacZ* expression. S150-2B carrying the *TRX2-lacZ* gene fusion was cultured in SD medium lacking uracil at 30 ºC until O.D600 ≈ 0.1-0.3. Cells were challenged with either H_2O_2 (0.49 mM) (▚) or BHA (0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 mM) (■) for 1 hour. NS (▩) was cells without treatment with any compounds. Pre-treated cells (□) were exposed to BHA (0.05, 0.1, 0.2 and 0.4 mM) prior for 1 hour then challenged with 0.49 mM H_2O_2 for 1 hour at 30 ºC. Data shown is the means ± S.D of three independent experiments. The asterisks (*) indicate the expression of the *TRX2-lacZ* of these treatments are significantly different from NS ($p < 0.05$). ^{xx} indicate the expression of the *TRX2-lacZ* of these treatments are significantly different from the expression of the *TRX2-lacZ* of 0.49 mM H_2O_2 treated cells ($p < 0.05$).

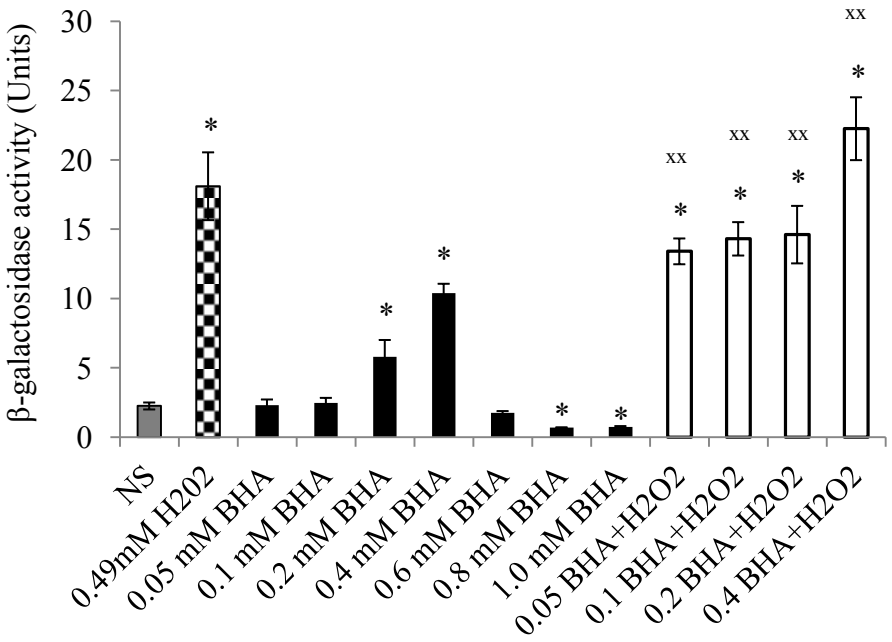


Figure 4.2 The effect of BHA on the induction of *SSA1-lacZ* expression. S150-2B carrying the *SSA1-lacZ* gene fusion was cultured in SD medium lacking uracil at 30 ºC until O.D600 ≈ 0.1-0.3. Cells were challenged with either H_2O_2 (0.49 mM) (▩) or BHA (0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 mM) (■) for 1 hour. NS (▩) was cells without treatment with any compounds. Pre-treated cells (□) were exposed to BHA (0.05, 0.1, 0.2 and 0.4 mM) prior for 1 hour then challenged with 0.49 mM H_2O_2 for 1 hour at 30 ºC. Data shown is the means ± S.D of three independent experiments. The asterisks (*) indicate the expression of the *SSA1-lacZ* of these treatments is significantly different from NS (p<0.05). ^{xx} indicate the expression of the *SSA1-lacZ* of these treatments are significantly different from the expression of the *SSA1-lacZ* of 0.49 mM H_2O_2 treated cells ($p < 0.05$).

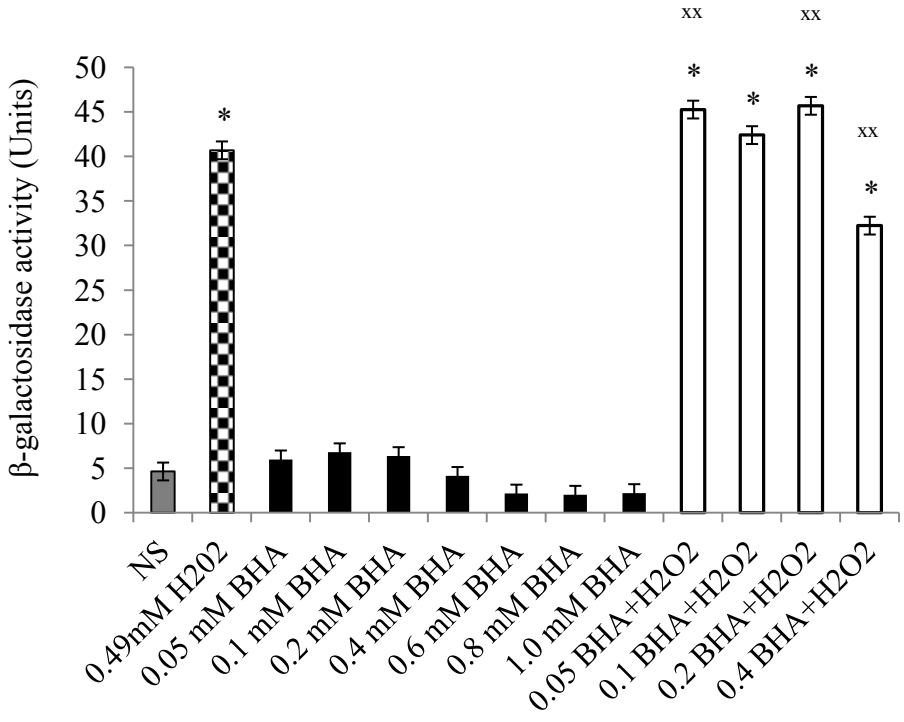


Figure 4.3 The effect of BHA on the induction of *GSH1-lacZ* expression. S150-2B carrying GSH*1-lacZ* was cultured in SD medium lacking uracil at 30 ºC until O.D600 ≈ 0.1-0.3. Cells were challenged with either H_2O_2 (0.49 mM) (▩) or BHA (0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 mM) (■) for 1 hour. NS (▩) was cells without treatment with any compounds. Pre-treated cells (□) were exposed to BHA (0.05, 0.1, 0.2 and 0.4 mM) prior for 1 hour then challenged with 0.49 mM H_2O_2 for 1 hour at 30 ºC. Data is shown as means of three independent experiments. The asterisks (*) indicate the expression of the *GSH1-lacZ* of these treatments is significantly different from NS ($p < 0.05$). ^{xx} indicate the expression of the *GSH1-lacZ* of these treatments are significantly different from the expression of the *GSH1-lacZ* of 0.49 mM H_2O_2 treated cells ($p < 0.05$).

CHAPTER 5 - GENETIC APPROACHES TO CHARACTERIZE BHA TOXICITY

With regard to the data in Chapter 3, pre-treatment of yeast cells with low concentration of BHA could induce protection towards toxic concentrations of both BHA and H_2O_2. However, exposure of BHA to non-pretreated yeast strain lacking Yap1 displayed higher cell viability than wild type (Figure 3.3). Also, cell viability of non-pretreated yeast strains lacking *SOD1*, *SOD2* and Msn2/Msn4 were not different from wild type strains after exposed to BHA (Figure 3.4 and Figure 3.5). Additionally, treatment yeast cells with various concentrations of BHA could not induce the expression of *TRX2* and *GSH1*, oxidant-regulated genes (Figure 4.1 and Figure 4.3). It looked like BHA might involve with non-oxidative route. A genetic approach was adopted to identify yeast genes responsible for BHA toxicity in order to gain an understanding of the mechanism of BHA toxicity. Mutagenesis is a technique used to generate mutants of interest in order to identify new genes. A classical mutagenesis screen can be carried out by treating cells with chemical mutagens such as; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and ethylmethane sulfonate (EMS). This technique can obtain an appropriate alteration in phenotype with no requirement for previous knowledge of the genes (Lawrence, 2002). In addition, the availability of mutant allows the isolation and identification of yeast genes by complementation of the phenotype by recombinant plasmids (Nasmyth and Reed, 1980).

In this chapter, the isolation of BHA hypersensitive mutants and the identification of yeast genes that responsible for BHA toxicity are described.

5.1 Isolation of BHA sensitive mutants

To obtain BHA sensitive mutants, *S. cerevisiae* wild types; S150-2B (*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-*Δ*1*) which was done in the initial work, the different mating types such as BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*) and BY4742 (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) were mutated by using ethyl methanesulfonate (EMS) and were used as parent strains to perform complementation test in the next step. As shown in Table 5.1, a total of 183 colonies unable to grow on YPD plate containing 0.5 mM or 0.8 mM BHA were identified by replica plating. These isolates were purified and the BHA sensitive phenotype confirmed by spot testing onto BHA supplemented plates.

Table 5.1 BHA sensitive mutants isolated from *S. cerevisiae* wild type strains

Strains	Number of mutants
S150-2B	57
BY4741	84
BY4742	52

Following spot testing, mutants that could not grow on YPD containing BHA plates were selected. The result illustrated that there were 17 mutants derived from S150-2B, twenty mutants of BY4741 and 39 mutants of BY4742 displaying various levels of BHA sensitivity (Figure 5.1). All of the mutations were recessive in nature which was confirmed by complementation analysis, if the BHA sensitive phenotype was recessive then complementation analysis would work. To determine whether two mutants were mutations in the same or different genes, complementation tests were performed. BHA sensitive mutants were chosen randomly to analyze for complementation. Representative *MAT***a** mutants were crossed with *MAT*α mutants. Diploids were then selected on SD+his+leu+ura plates and tested for BHA sensitivity. The results showed that diploid with parents strains were able to grow on both YPD supplemented with 0.5 and 0.8 mM BHA (data not shown). In addition, all of the diploid mutants could grow on YPD+0.5 mM BHA (data not shown) and some of them were able to grow on YPD plates containing 0.8 mM BHA (Table 5.2) whereas all of the haploids were sensitive to 0.8 mM BHA. Diploids that displayed a mutant phenotype (no growth on YPD+BHA plates) were classified as being in the same complementation group and could be defined as alleles of the same gene. Pairs of mutants that exhibited wild type phenotype (good growth on YPD+BHA plates) were classified in being in different complementation groups. In this experiment *MAT***a** mutants and *MAT*α mutants were chosen randomly to perform cross mating. Example of cross mating and diploid phenotypes is shown in Table 5.2. The data show that four mutants (MS-54, MB2-4, MB2-22 and MB2-43) were grouped in the same complementation group, implying that they are mutated in the same gene. MS-24, MS-39, MS-10 and MB2-36 were classified in different complementation groups, indicating that these mutants might be in different genes. These experiments showed that the mutations defined 5 complementation groups (Table 5.3, set 1). Example of complementation groups of other mutants are also shown in Table 5.3.

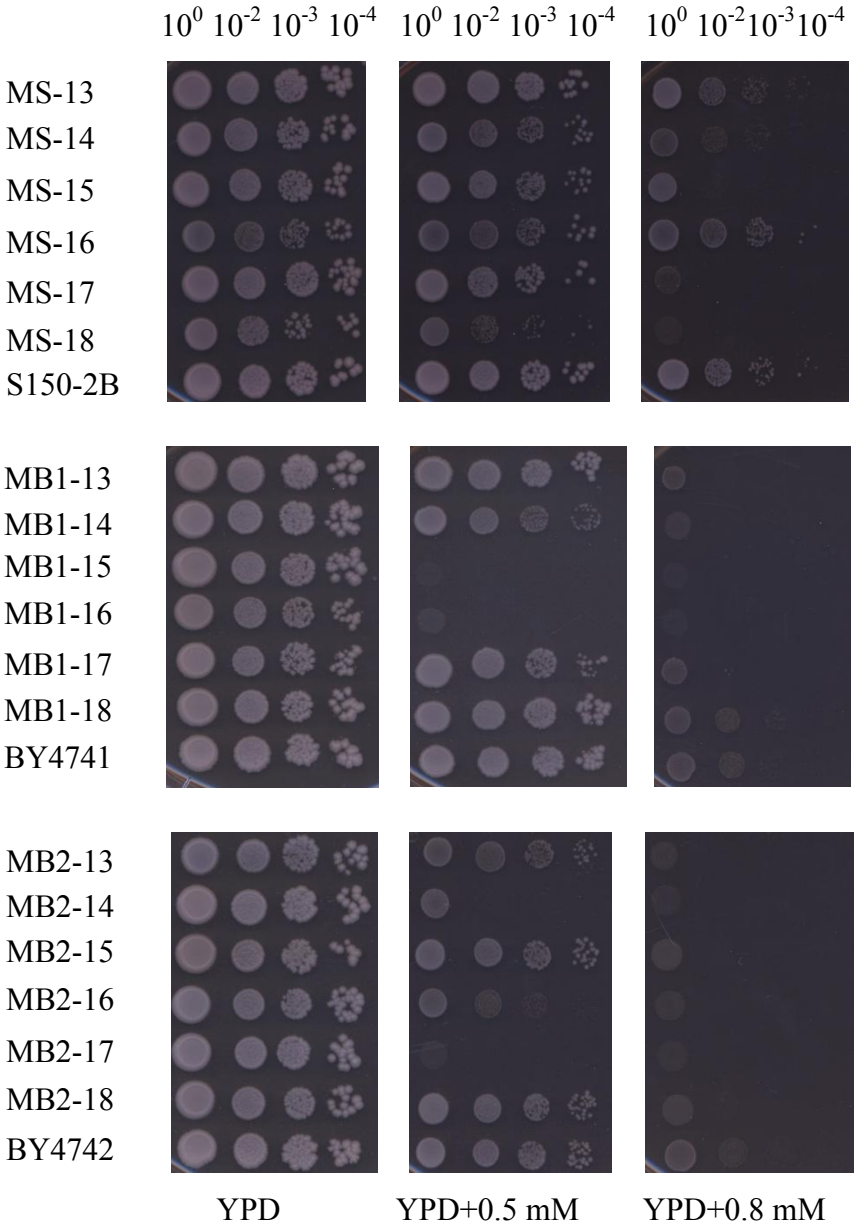


Figure 5.1 Example of spot test showing BHA sensitivity of mutants derived from *S. cerevisiae* strains S150-2B, BY4741 and BY4742. Serial dilutions of each mutant were spotted onto YPD agar and YPD containing 0.5 or 0.8 mM BHA. Plates were incubated at 30°C for 4 days.

Table 5.2 Diploid phenotypes on YPD containing 0.8 mM BHA plates

MATα Strains	*MAT***a** Strains			
	MS-24	MS-39	MS-54	MS-10
MB2-4	+	+	-	+
MB2-22	+	+	-	+
MB2-43	+	+	-	+
MB2-36	+	++	++	+

Scoring: ++ represents as good growth, + represents a moderate growth and – is no growth

Table 5.3 Classes of mutant complementation groups

Complementation groups	Mutants derived from wild type strains	
	S. cerevisiae S150-2B	*S. cerevisiae* BY4742
Set 1* (8 mutants)		
Group 1	MS-54	MB2-4
		MB2-22
		MB2-43
Group 2	MS-24	
Group 3	MS-39	
Group 4		MB2-36
Group 5	MS-10	
Set 2 (7 mutants)		
Group 1	MS-18	MB2-4
	MS-50	
Group 2	MS-12	
Group 3		MB2-42
Group 4		MB2-36
Group 5	MS-24	

* Set no. refers to random cross mating of *MAT***a** mutants and *MATα* mutants

Table 5.3 Classes of mutant complementation groups (continue)

Complementation groups	Mutants derived from wild type strains	
	S. cerevisiae S150-2B	*S. cerevisiae* BY4742
Set 3 (9 mutants)		
Group 1	MS-54	MB2-44
	MS-50	MB2-52
Group 2		MB2-59
Group 3		MB2-64
Group 4	MS-27	
Group 5	MS-31	
Group 6	MS-39	

* Set no. refers to random cross mating of *MAT***a** mutants and *MAT*α mutants

5.2 Cloning of the genes responsible for the BHA sensitivity phenotype by complementation

To identify the genes responsible for the BHA sensitivity phenotype, a yeast genomic library (constructed in either YEp13 or YEp24) was transformed into the mutant strains. Transformants carrying YEp 13 plasmid were selected on SD-leu plates or transformants carrying YEp24 plasmid were selected on SD-ura and also their ability to grow on YPD+BHA plates was tested. Representative mutants from each of the five complementation groups were chosen for transformation with the plasmid YEp13 genomic library. Leu^+ transformants were selected by plating out onto SD agar plates lacking of leucine. These transformants were then screened for the BHA sensitivity phenotype by spot testing on YPD agar plates containing 0.5 and 0.8 mM BHA. After screening for complementation of the BHA sensitivity phenotype, there were only 6 transformants, derived from MB2-36, MS-24, MS-10, MS-39, MS-50 and MB1-11 mutants, that could grow on YPD containing both 0.5 mM and 0.8 mM BHA (Table 5.4). Most of the transformants were unable to grow on YPD supplemented BHA. This might be due to instability of plasmid.

In addition to the YEp13 genomic library, a YEp 24 genomic library was transformed into some of BHA sensitive mutants in order to maximize the possibility of obtaining complementing clones. Ura^+ transformants were selected onto SD plates without uracil and then screened for the BHA sensitive phenotype by spot testing. Growth of transformants carring YEp24 plasmids with genomic DNA inserts is shown in

Table 5.5. The results illustrated that two transformants of the mutants (MB2-17 and MS-54) were capable of growth on YPD plates containing 0.5 and 0.8 mM BHA.

To prove that the strains contained plasmids complementing the BHA sensitivity phenotype, the plasmids were isolated and amplified in *E.coli*. Plasmids pYCC1, pYCC2, pYCC3, pYCC4, pYCC5, pYCC6, pYCC7 and pYQZ were isolated and purified from the transformants of MB2-36, MS-24, MS-10, MS-39, MS-54, MS-50, MB1-11 and MB2-17 respectively. These plasmids were amplified in *E.coli* and re-transformed to each of the original yeast mutants. The re-transformed yeasts were then tested for their BHA sensitivity, Figure 5.2 and Figure 5.3. Seven of eight plasmids were able to restore the ability of the mutants to grow on YPD+0.8 mM BHA plates, complementing the BHA sensitive mutations. However, strain MB1-11 was unable to grown on YPD containing 0.8 mM BHA after being retransformed with plasmid pYCC7. This might be due to there being more than one plasmid in the original transformant (Ausubel, et al., 2003). Alternatively it could also be that the plasmid probably is instable.

Table 5.4 Growth of transformants isolated from the YEp13 genomic DNA library

Strains	SD-leu	YPD	YPD+0.5 mM BHA	YPD+0.8 mM BHA
Original mutants				
MB2-36	-	++	-	-
MS-10	-	++	++	-
MS-24	-	++	++	-
MS-39	-	++	++	-
MS-50	-	++	++	-
MB1-11	-	++	++	-
Wild types				
S150-2B	-	++	++	+
BY4741	-	++	++	+
BY4742	-	++	++	-
Transformants				
MB2-36+YEp13 genomic library	++	++	++	-
MS-10+YEp13 genomic library	++	++	++	+
MS-24+YEp13 genomic library	++	++	++	+
MS-39+YEp13 genomic library	++	++	++	+
MS-50+YEp13 genomic library	++	++	++	+
MB1-11+YEp13 genomic library	++	++	++	+
Wild type transformants				
S150-2B+YEp13 genomic library	++	++	++	+
BY4741+YEp13 genomic library	++	++	++	-
BY4742+YEp13 genomic library	++	++	++	-

Scoring: ++ represents as good growth, + represents a moderate growth and – is no growth

Table 5.5 Growth of transformants isolated from the YEp24 genomic DNA library

Strains	SD-ura	YPD	YPD+0.5 mM BHA	YPD+0.8 mM BHA
Original mutants				
MB2-4	-	++	+	-
MB2-17	-	++	-	-
MB2-42	-	++	-	-
MS-54	-	++	+	-
Wild types				
S150-2B	-	++	++	-
BY4742	-	++	++	-
Transformants				
MB2-4+YEp24 genomic library	++	++	-	-
MB2-17+YEp24 genomic library	++	++	+	-
MB2-42+YEp24 genomic library	++	++	-	-
MS-54+YEp24 genomic library	++	++	++	+
Wild type transformants				
S150-2B+YEp24 genomic library	++	++	++	++
BY4742+YEp24 genomic library	++	++	++	+

Scoring: ++ represents as good growth, + represents a moderate growth and – is no growth

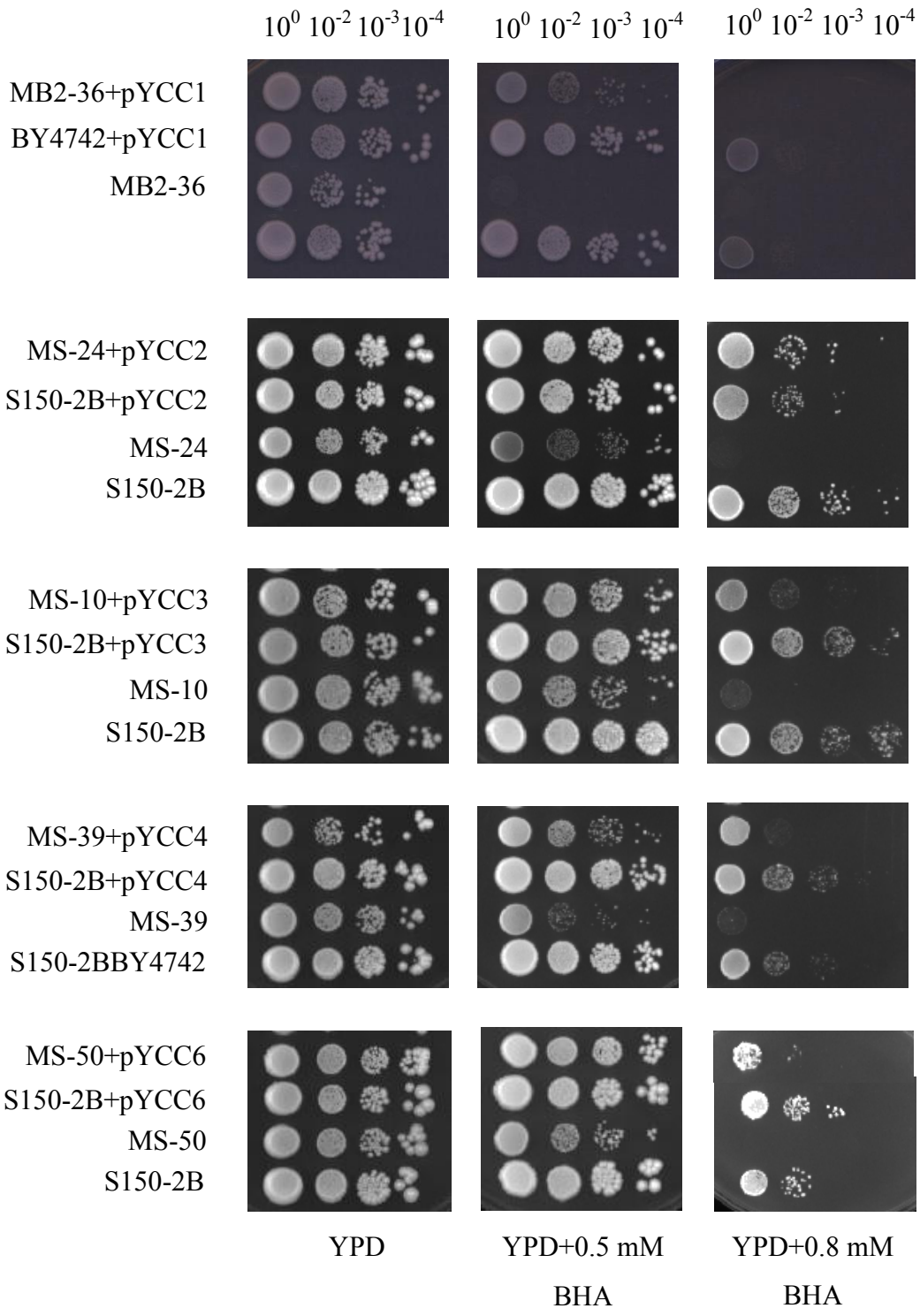


Figure 5.2 BHA sensitivity tests of MB2-36, MS-24, MS-10, MS-39 and MS-50 transformants. The mutants (MB2-36, MS-24, MS-10, MS-39 and MS-50) were retransformed with plasmids (pYCC1, pYCC2, pYCC3, pYCC4 and pYCC6) and the re-transformants screened for BHA sensitivity. Each dilution was spotted onto YPD agar and YPD containing 0.5 or 0.8 mM BHA. Plates were incubated at 30 °C for 4 days.

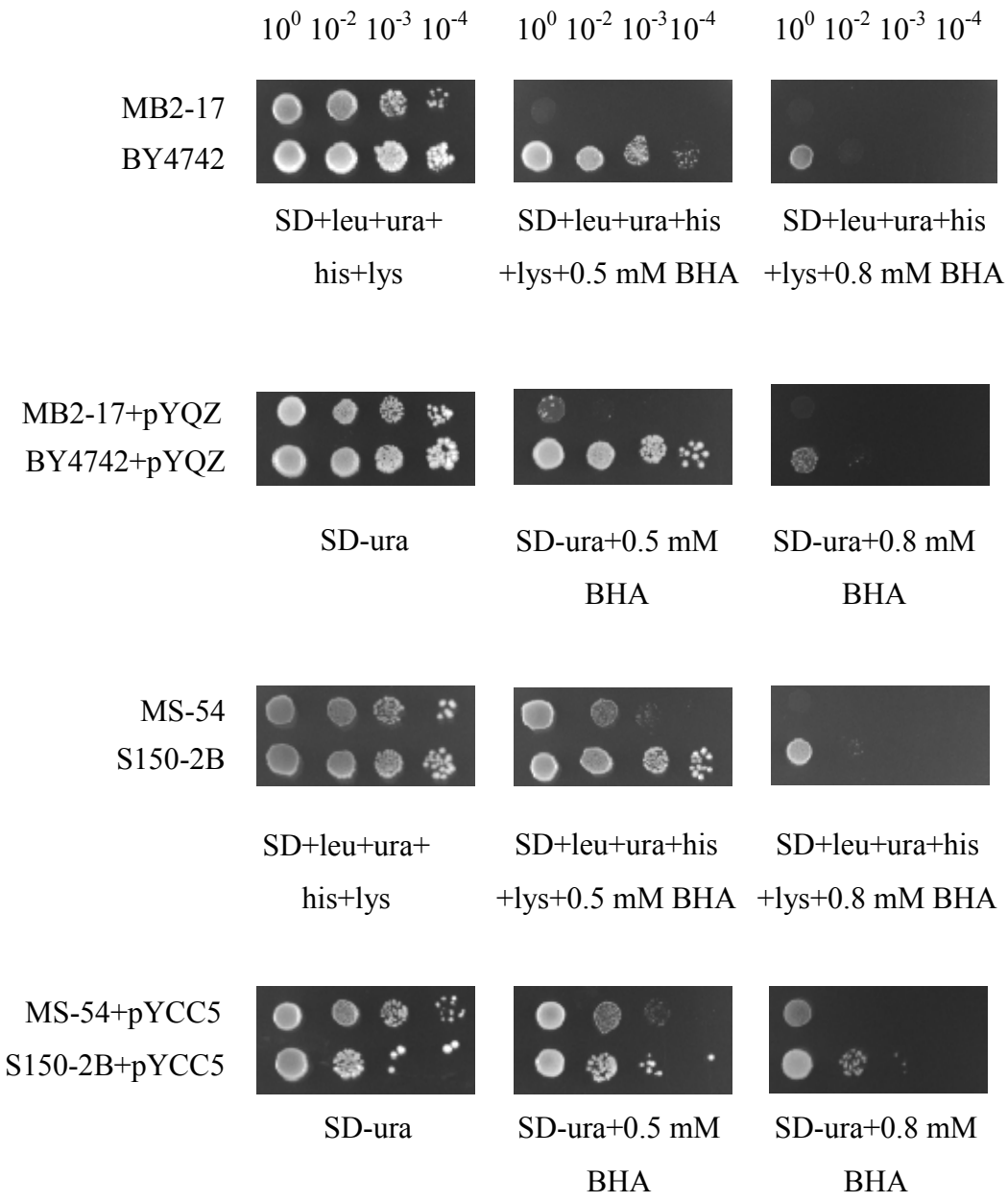


Figure 5.3 BHA sensitivity tests of the MB2-17 and MS-54, transformants. The mutants (MB2-17 and MS-54) were retransformed with plasmids (pYQZ and pYCC5) and the re-transformants screened for BHA sensivity. Each dilution was spotted onto SD agar and SD-ura containing 0.5 or 0.8 mM BHA. Plates were incubated at 30 °C for 4 days.

5.3 Gene identification

Having shown in previous section, there are 5 plasmids of LEU2 derivative plasmid (pYCC1, pYCC2, pYCC3, pYCC4 and pYCC6) and 2 plasmids of URA3 derivative plasmid (pYCC5 and pYQZ) capable of complementing the BHA sensitive mutations, these plasmids were purified and were then analyzed by restriction enzyme and gel electrophoresis (Appendix B.). In addition, DNA sequencing was performed in order to identify the genes which were inserted in the genomic DNA plasmid. The oligonucleotide 5'-TCACTATGGCGTGCTGCTAGCGCT-3' (ox164) or 5'-TGCGT TGATGCAATTTCTATG-3' (ox517) was used as forward primer at the *Bam*HI site flanking. For the reverse primer was 5'-CTGCCACCATACCCACGC CGAAAC-3' (ox165). The DNA insert of each complemented plasmids was sequenced and compared with *Saccharomyces* genome database (SGD) by performing BLAST searches. These results found that LEU2 derivative plasmids; pYCC1, pYCC3 and pYCC6 have inserts from regions of chromosome II, 2 micron plasmid fragment and chromosome XIII respectively. The genes found in these inserts and the sizes of the plasmids are described in Table 5.6. However, the plasmids pYCC2 and pYCC4 have no sequences, therefore BLAST searches were not carried out. In the case of the URA3 derivative plasmids; pYCC5 plasmid has an insert on chromosome XVI containing genes; *KRE6* and *GPH*. Also, an insert from chromosome V was found in a plasmid pYQZ. Details of the insert found in this plasmid are described in Table 5.6.

Table 5.6 Genes found in the inserts

Plasmid	Size of plasmid (kbp)	Chromosome	Gene
pYCC1	20.08	II	*UBS1*, *TYR1*, *POP7*, *PEX32*, *SSE2*
pYCC3	15.36	2μ plasmid	
pYCC5	24.24	XVI	*KRE6*, *GPH1*
pYCC6	18.15	XIII	*NGL3*, *YML119w*, *NDI1*, *GTR1*
pYQZ	15.82	V	*PMP2*, *GTT3*, *EAF5*, *YEL020c*

To determine which of these genes was responsible for complementing the BHA sensitivity, strains deleted for the various genes purchasing from EUROSCRAF were spotted onto YPD agar supplemented with 0.5 mM and 0.8 mM BHA. Spot tests showed that the phenotype of strain with the *TYR1* gene deleted was similar to that of the original mutant (MB2-36). They could not grow on YPD+0.5 mM BHA plates whereas deletions of other genes present on the chromosome II insert were capable of growing (Figure 5.4). Additionally, strains deleted for the *KRE6* and *GPH1*genes on chromosome XVI were unable to grow on SD+0.8 mM BHA plates. Plasmid pYCC5 carried these genes and was able to complement the mutation in strain MS-54 (Figure 5.5). These findings indicated that the proteins coded for by the genes *TYR1*, *KRE6* and *GPH1* play some role in the cells response towards BHA. Given that there were two genes in plasmid pYCC5 isolated from the mutant MS-54 that responded to BHA sensitivity, subsequent investigation would determine which of these genes (*KRE6* or *GPH1*) are responsible for the BHA sensitivity. Sub-cloning of the plasmid pYCC5 would be applied. This plasmid would be cut with restriction enzymes and the desired band would be separated by gel electrophoresis. The desired fragment containing of *KRE6* or *GPH1* would insert into a new vector. Subcloned plasmids would be maintained in *E. coli*, then purified and transformed back into the original mutant (MS-54). Transformants would be then tested for complementing BHA sensitivity by spot testing onto YPD agar plates containing of 0.5 mM and 0.8 mM BHA or SD plates supplemented with 0.5 mM and 0.8 mM BHA.

With regard to spot tests of the stains deleted for the genes present on the chromosome V and XIII insert containing plasmids, they failed to complement the MB2-17 (pYQZ) and MS-50 (pYCC6) mutants (data not shown). It was noticed that phenotype of MS-50+pYCC6 transformant could no longer grow on YPD+0.8 mM plate when a repeat spot test was carried out. This may be caused by instability of the plasmid resulting in the loss of transformed phenotype (Zhang, et al., 1996).

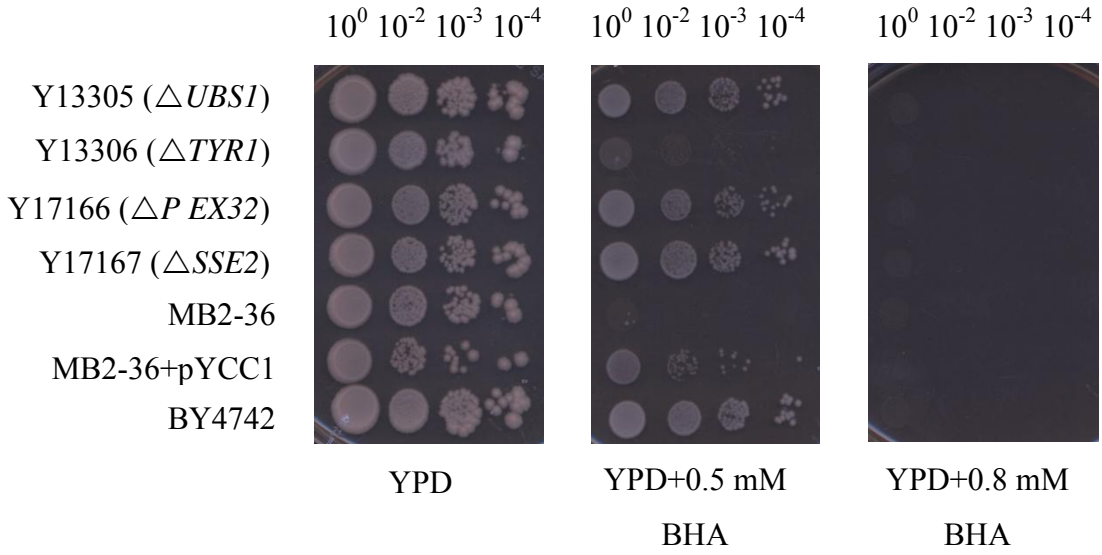


Figure 5.4 BHA sensitivity phenotype of gene deletion strains and plasmid pYCC1 on YPD agar and YPD agar containing 0.5 or 0.8 mM BHA. Plate was incubated at 30ºC, 4 days.

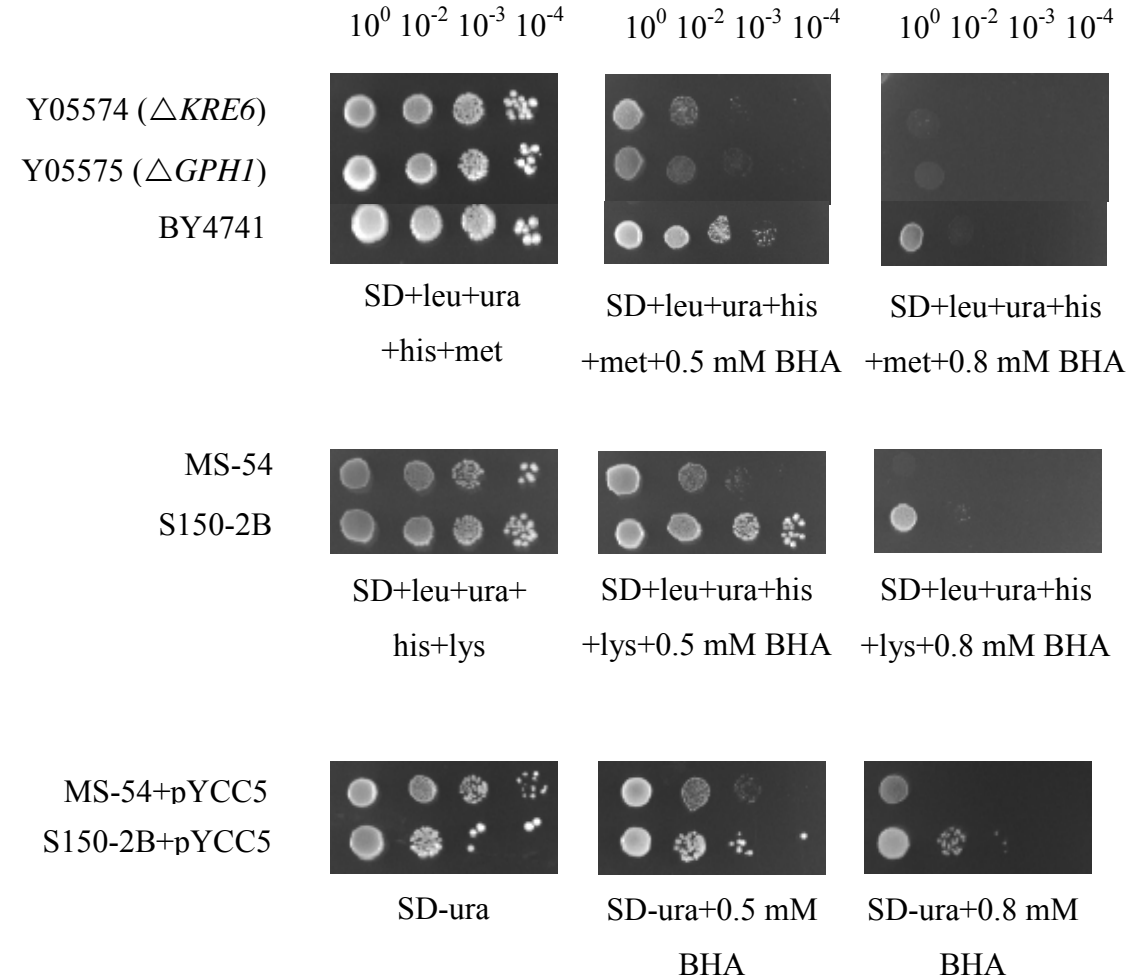


Figure 5.5 BHA sensitivity phenotype of gene deletion strains and plasmid pYCC5 on SD agar and SD agar containing 0.5 or 0.8 mM BHA. Plates were incubated at 30ºC, 4 days.

5.4 Effect of plasmid on BHA sensitive phenotype

During these experiments it was found that the phenotype of some the transformants had reverted or were unstable. It has been revealed that there were many factors such as plasmid copy number, selective markers, properties of host cells and medium formulation affected plasmid stability (Zhang, et al., 1996). In order to determine whether the copy numbers of the LEU2 gene affect the BHA phenotype, phenotype of the transformants carried pYCC3 complemented plasmid (LEU2 derivative plasmid) were compared with the transformants carried YEp13 (high copy number plasmid) and pRS415 (low copy number plasmid). A representative of BHA sensitive mutants (MS-10) was transformed with plasmids pYCC3, YEp13 and pRS415. LEU^+ transformants were then selected onto SD agar plates without leucine. These transformants were spotted onto YPD agar supplemented with 0.5 mM and 0.8 mM BHA in order to compare their BHA sensitive phenotypes. The results are shown in Table 5.7. Phenotype of MS-10 carried pYCC3 and YEp13 exhibited good growth on YPD+0.8 mM BHA, whereas MS-10 carried pRS415 was unable to grow on YPD+0.8 mM BHA. These findings implied that the copy number of the plasmid can affect the transformed phenotype. This because of low copy number might be involved partitioning of plasmid which lead to plasmid instability and loss of desired product (Zhang, et al., 1996). However, different results were observed in different host cells. Transformed phenotype of MS-24 carried plasmid YEp13 exhibited no growth on YPD+0.8 mM BHA (data not shown). This is consistent with the research of Zhang, et al. (1996), reporting that different host mutants might affect plasmid partitioning and replication resulting in displaying of different transformant phenotypes even a particular plasmid was transformed.

Table 5.7 Growth of mutants carrying various plasmids

Strains	SD-leu	YPD	YPD+0.5 mM BHA	YPD+0.8 mM BHA
MS-10	-	++	++	-
S150-2B	-	++	++	+
S150-2B (△2μ)	-	++	++	+
pYCC3 transformants				
MS-10+pYCC3	++	++	++	+
S150-2B+pYCC3	++	++	++	++
YEp13 transformants				
MS-10+YEp13	++	++	++	+
S150-2B+YEp13	++	++	++	++
pRS415 transformants				
MS-10+pRS415	++	++	++	-
S150-2B+pRS415	++	++	++	+

Scoring: ++ represents as good growth, + represents a moderate growth and – is no growth

5.5 Characterization of BHA sensitive mutants

It has been reported that amino acids can result in cell protection against stress (Ball, et al., 1986; Lupo, et al., 1997). Lupo, *et al* (1997) revealed that cell viability of a *S. cerevisiae* mutant which had lost the ability to synthesize tyrosine was decreased and also showed higher sensitivity to hydrogen peroxide. To examine whether or not the *bha* mutants were auxotrophic for tyrosine, the mutants were grown in SD medium supplemented with the necessary amino acids and additional tyrosine or phenylalanine (as the *TYR1* gene is transcriptionally stimulated by the presence of phenylalanine). The initial OD_{600} of cell cultures was 0.1 and the cultures were then incubated at 30°C with shaking for 72 hours. One millilitre of the cultures was taken to measure cell growth at OD_{600} using a spectrophotometer. Figure 5.6 and Figure 5.7 show the growth of mutant (MB2-36) and wild type (BY4742) in SD medium supplemented with different amino acids. These observations revealed that growth of MB2-36 was significant lower than wild type (BY4742) when they were cultured in SD+his+leu+lys+ura and SD+his+leu+lys+ura+phe (in the absence of tyrosine). Whereas the growth capability of MB2-36 was restored to wild type levels when

incubated in SD medium supplemented with tyrosine, showing that the MB2-36 mutant was impaired in tyrosine biosynthesis. As for the other strains, Figure 5.8 - Figure 5.10 demonstrated that MS-24 and MS-50 mutants were able to grow in SD medium, SD+tyrosine and SD+phenylalanine. However, their growth were lower than wild type (S150-2B), indicating that tyrosine synthesis might not be affecting growth of these mutants.

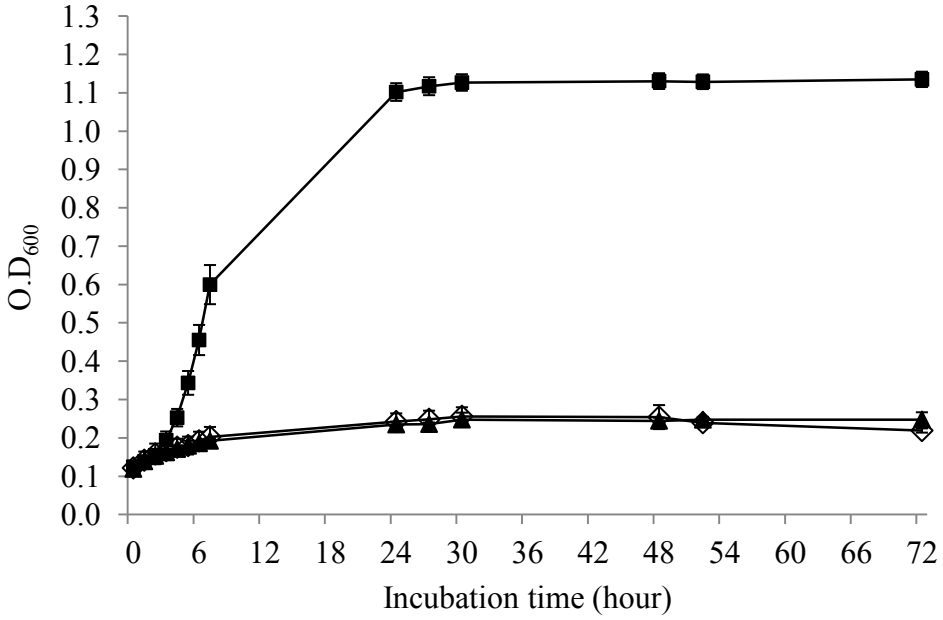


Figure 5.6 The effect of tyrosine and phenylalanine on the growth of mutant MB2-36. MB2-36 was cultured in SD medium+his+leu+lys+ura (◇), SD medium+ his+leu+lys +ura+tyr (■) and SD medium+ his+leu+lys+ura+phe (▲). Cells were incubated with shaking at 30ºC for 72 hours. Values are the mean ± the SD of three independent experiments.

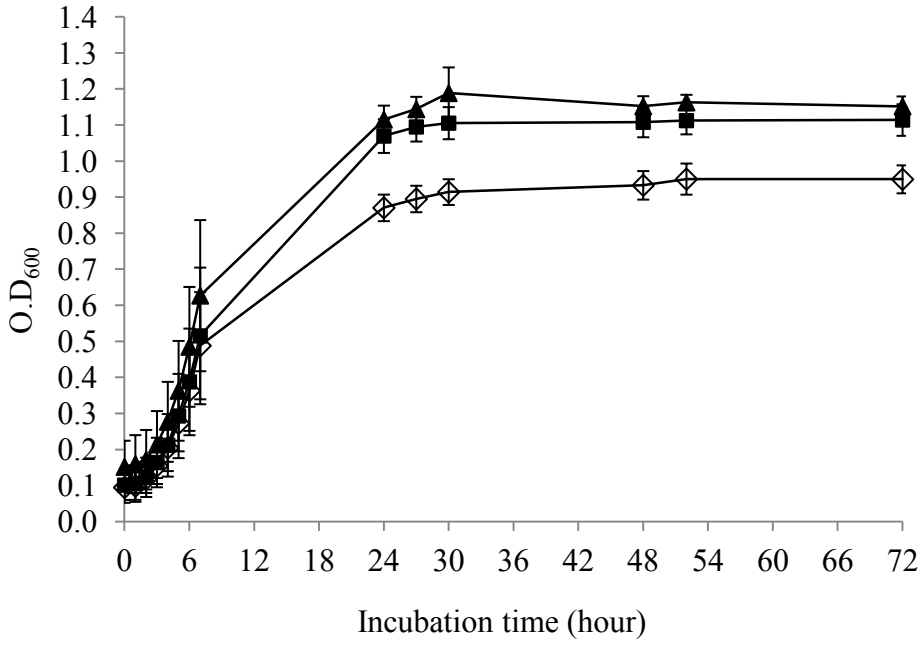


Figure 5.7 The effect of tyrosine and phenylalanine on the growth of BY4742. The cultures were grown in SD medium+his+leu+lys+ura (◇), SD medium+his+leu+lys +ura +tyr (■) and SD medium+his+leu+lys+ura +phe (▲). Cells were incubated with shaking at 30ºC for 72 hours. Values are the mean ± the SD of three independent experiments.

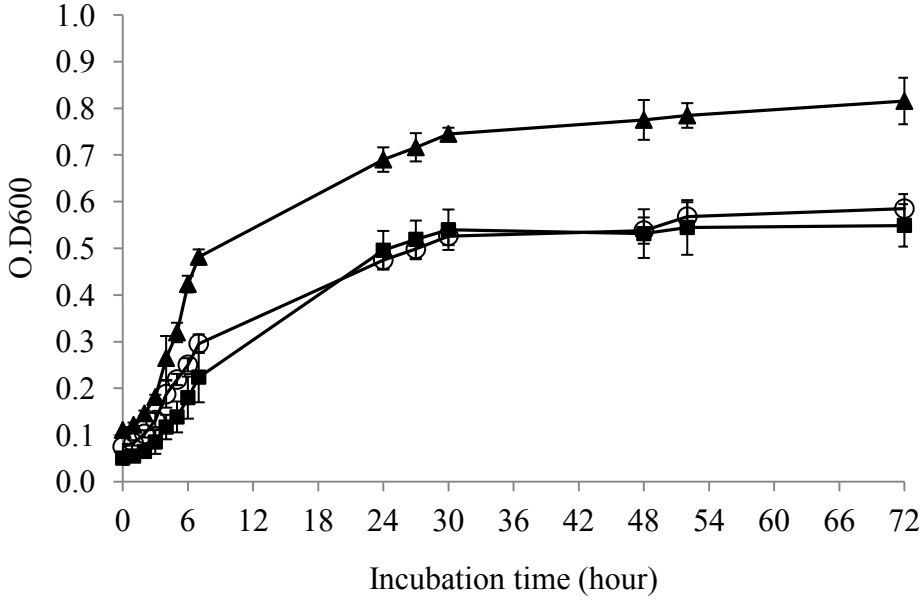


Figure 5.8 The growth of *S. cerevisiae* strain S150-2B (▲) and the mutants (MS-24 (■) and MS-50 (○)) in SD medium. Cells were incubated with shaking at 30ºC for 72 hours. Values are the mean ± the SD of three independent experiments.

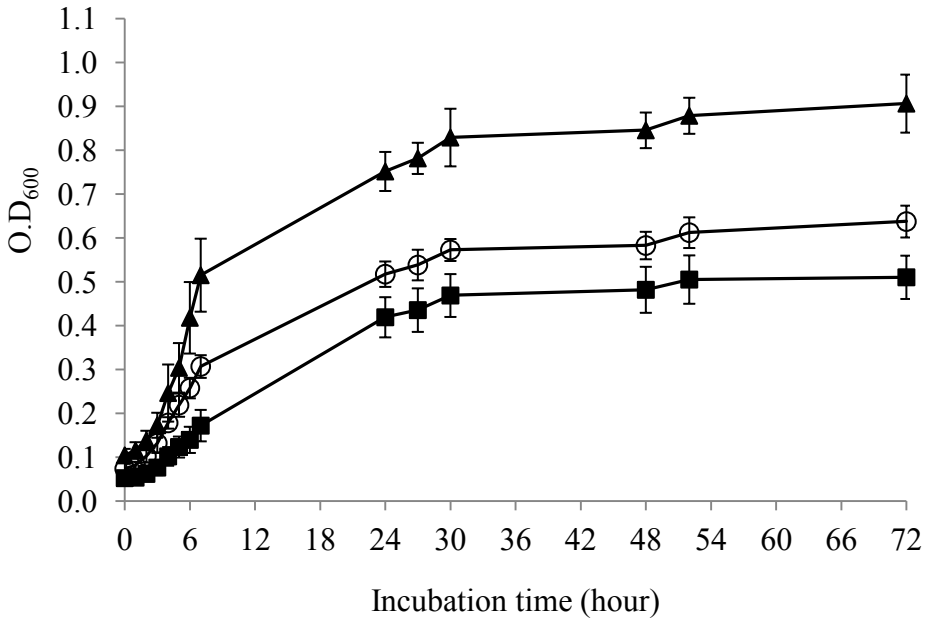


Figure 5.9 The effect of tyrosine on the growth of *S. cerevisiae* strain S150-2B (▲) and the mutants (MS-24 (■) and MS-50 (○)). These cultures were inoculated SD medium+ his+leu+trp+ura+tyr. Cells were incubated with shaking at 30ºC for 72 hours. Values are the mean ± the SD of three independent experiments.

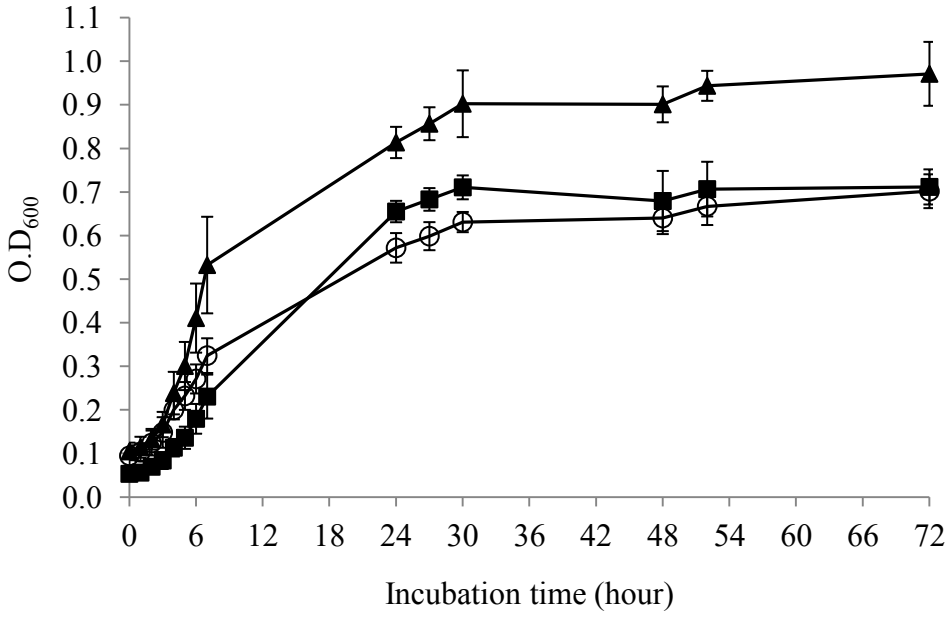


Figure 5.10 The effect of phenylalanine on the growth of *S. cerevisiae* strain S150-2B (▲) and the mutants (MS-24 (■) and MS-50 (○)). These strains were cultured in SD medium+ his+leu+trp+ura+phe. Cells were incubated with shaking at 30ºC for 72 hours. Values are the mean ± the SD of three independent experiments.

CHAPTER 6 – DISCUSSION AND CONCLUSION

6.1 BHA and yeast adaptive responses

It has been reported that BHA and TBHQ (*tert*-butylhydroquinone) can stimulate superoxide production in rat liver microsomes. Also, the generation of oxygen radicals by BHA and TBHQ leads to induction of AP-1 (an antioxidant responsive factor) and glutathione S-transferase (GST) Ya gene expression in mammalian cells (Kahl, et al., 1989; Pinkus, et al., 1996). In this study, therefore the question has been addressed whether BHA functions by acting as a pro-oxidant to induce gene expression which is responsible for oxidative stress. BHA was used as a stressor and applied to *S*. *cerevisiae* strains and the sensitivity of yeast cells to this antioxidant was determined. In general, when living cells, such as yeast, are challenged with different environmental stress conditions, they are able to adjust their metabolism and cellular processes in order to maintain their growth in the new conditions. This phenomenon is a common response to stress. As presented in chapter 3 (section 3.1), *S*. *cerevisiae* strain S150-2B was found to be sensitive to BHA. Survival of the yeast cells dramatically decreased when the concentrations of BHA was increased (Figure 3.2 and Figure 3.3), demonstrating that BHA was toxic to the yeast cells. This might be due to BHA inducing oxidative DNA damage (Ali and Suzuki, 2012). Treatment of the yeast cells with a sub-lethal dose of BHA (0.2 mM) resulted in the cells becoming tolerant to higher doses of BHA (Figure 3.3), indicating that *S*. *cerevisiae* possessed adaptive stress response toward BHA. This result is consistent with several studies which showed that yeast possesses several adaptive stress responses (Jamieson, 1992; Flattery-O'Brien, et al., 1993; Izawa, et al., 1995; Jamieson, et al., 1996; Jamieson, 1998; Biryukova, et al., 2008). Pre-treatment of yeast *Candida albicans* and *S.cerevisiae* (S150-2B) with low concentrations of either H_2O_2 or menadione (a superoxide generator) induced protection against high concentration of these oxidants (Jamieson, 1992; Jamieson, et al., 1996). The yeast cells produced stress proteins after sensing a low dose of stressor compound, resulting in resistance toward the lethal dose of the same stressor (Mager and Hohmann, 1997). Izawa, et al. (1995) reported that *S*. *cerevisiae* could adapt to H_2O_2 stress by increasing glutathione content after treatment with 0.2 mM H_2O_2.

Importantly, as shown in chapter 3, BHA treated *S*. *cerevisae* strains became cross-adapted which respond to H_2O_2, as pre-treatment yeast cells with 0.2 mM BHA led to cells becoming tolerant to higher concentrations of H_2O_2 (Figure 3.6 – Figure 3.8). This

suggests that the BHA response is either identical to the H_2O_2 response or might provide overlapping defence for protecting against H_2O_2. There is a precedence for overlapping stress responses, it has been reported that yeast could mount cross-protection to specific stress responses or a general stress response (Mager and Hohmann, 1997). Flattery-O'Brien, et al. (1993) showed that pre-treated yeast cells with heat stress acquired resistance to high concentration of menadione, however menadione pre-treatment could not induce heat resistance. Lu, et al. (2005) reported that *S. cerevisiae* exhibited cross-adaptation between hyperosmotic stress and oxidative stress. Pre-treatment of *S. cerevisiae* cells with mild concentration of hyperosmotic stress (1% KCl) resulted in increased levels of antioxidants (glutathione, catalase and superoxide dismutase) and also increased resistance to lethal concentration of H_2O_2.

6.2 BHA and oxidative stress response

Having shown that BHA was toxic to yeast cells and low concentrations of the antioxidant (0.2 mM BHA) could induce protection towards toxic levels of both BHA and H_2O_2 (section 3.2 and section 3.3), several questions arose: did BHA toxicity result from oxidative stress and, was the BHA adaptive response actually the oxidative stress response? Previous studies have reported that yeast cells can develop several antioxidant defence system such as enzymatic defence system (superoxide dismutases (SODs), catalase, and glutathione peroxidase) and non-enzymatic system (small molecules including glutathione, vitamin E and ascorbate) to protect themselves against oxidants (Santoro and Thiele, 1997; Jamieson, 1998; Herrero, et al., 2008). A number of researches have illustrated that the Yap1, Skn7 and Msn2/4 transcription factors are key regulators in response to oxidative stress (Stephen, et al., 1995; Lee, et al., 1999; Estruch, 2000; Moradas-Ferreira and Costa, 2000; Herrero, et al., 2008; Morano, et al., 2012). Genes regulated through Yap1 such as *TRX2*, *GSH1*, *SOD1* and *SOD2* were activated by H_2O_2 and superoxide anion (Estruch, 2000). Moreover, Lee, et al. (1999) reported that Skn7 transcription factor cooperates with Yap1 to activate *TRX2* and *TRR1* gene expression in response H_2O_2 stress, Yap1 is a major transcription factor for H_2O_2 stress response. The Msn2/4 proteins are zinc finger transcription factors and bind to the stress response element (STRE) which plays a role in resistance to various forms of stress. It has been reported that yeast genes, for example, *CCT1, DDR2, HSP12, HSP26* and *HSP104* are controlled by Msn2/4 through STRE-mediated induction (Martinez-Pastor, et al., 1996; Estruch, 2000; Amorós and Estruch, 2001). In this section, therefore, the effect of BHA and H_2O_2 on oxidative stress response was focused. Yeast

strains lacking Yap1, Sod1, Sod2 and Msn2/Msn4 were challenged with BHA and H_2O_2 in order to determine whether or not BHA was acting solely as a pro-oxidant in inducing protection against oxidative stress, in addition, the effect of BHA on the expression of known oxidant regulated genes (*TRX2*, *SSA1* and *GSH1*) was also determined.

BHA sensitivity of strain lacking Yap1 was lower than wild type (Figure. 3.3). Yeast strains lacking the Msn2/Msn4 transcription factor or lacking *SOD1* and *SOD2* genes were as sensitive to BHA as wild types (Figure 3.4 and Figure 3.5). However, the strains lacking of Yap1, Sod1, Sod2 and Msn2/Msn4 still acquired adaptive response to severe BHA stress after pre-treatment with low concentration of BHA (Figure 3.3-Figure 3.5). This suggests that BHA toxicity might not involve the classic oxidative stress response. It has been reported that *TRX2* and *GSH1* genes play a role in protection of oxidative stress by synthesis non-enzymatic antioxidant molecules like thioredoxin and glutathione (Santoro and Thiele, 1997). The expression of *TRX2* gene (encoding thioredoxin2), and *GSH1* gene (encoding γ-glutamylcysteine synthetase) are induced by H_2O_2 and superoxide anions, respectively (Stephen, et al., 1995; Santoro and Thiele, 1997). In addition, *SSA1*, encoding an HSP70 isoform was found to be involved with oxidative stress, this heat shock gene is induced by H_2O_2 (Jamieson, et al., 1994). Stephen, et al. (1995) illustrated that the H_2O_2-mediated induction of *SSA1* gene expression required *YAP1*. H_2O_2 could not induce *SSA1* gene expression in mutant strain lacking *yap1*. Raitt, et al. (2000) also reported that *SKN7* is required to induction of *SSA1* expression in response to hydrogen peroxide. They suggested that Skn7 transcriptional regulator interact with Hsf1, heat shock factor, in response to oxidative stress. Here, the assumption was that if BHA could act as a weak oxidant, the expression of these genes in the yeast cells would be elevated by BHA treatment alone. The data in Chapter 4 showed that the expression of the *TRX2* and *GSH1*genes in BHA-treated cells were not different from non-stress cells (Figure 4.1 and Figure 4.3). Interestingly, in the cells that were treated with 0.04 mM BHA showed high levels of expression of *SSA1* (Figure 4.2). This is possible that BHA could be mediated induction of *SSA1* gene expression, implyting that heat shock response might involve with BHA stress response. These results indicated that BHA was not able to activate expression of key oxidant-induced genes. The effect of BHA on the expression of *SSA1* would merit further study in order to know whether or not BHA could induce the heat shock stress

response. Moreover, the antioxidant enzyme levels such as catalase, glutathione reductase and thioredoxin reductase could be measured.

With regard to H_2O_2 treatment, the data showed that non-adapted cells of the strain lacking Yap1 were more sensitive to H_2O_2 than wild type cells (Figure 3.6), consistent with several studies, reporting that the Δ *yap1* mutant had a defect in H_2O_2 tolerance. These results confirm that Yap1 plays a role in the induction of the H_2O_2 adaptive response (Stephen, et al., 1995; Ouyang, et al., 2011). Also, strains lacking in Msn2/Msn2 displayed a higher sensitivity to H_2O_2 compared with the wild type (Figure 3.8). This is because H_2O_2 activates the Msn2/Msn4 transcription factor in response to a variety of stresses, such as oxidative stress. Thus, the Δ msn2/msn2 mutant showed a defect in response to H_2O_2, consistent with that found by Berry and Gasch (2008). In contrast, non-pretreated strains lacking the *SOD1* and *SOD2* genes exhibited wild type levels of H_2O_2 sensitivity (Figure 3.7). However, pre-treatment of these strains with 0.2 mM BHA allowed cells to grow in a higher concentration of H_2O_2, showing that BHA can participate in cross-protection against H_2O_2. It is possible that BHA might induce the expression of genes, which are regulated by the general stress transcription factor, in a way that is independent of Msn2/4. In addition the results show that the expression of *TRX2*, *SSA1* and *GSH1* were induced by H_2O_2, consistent with Stephen, et al. (1995), with expression levels in H_2O_2-treated cells being significantly higher than that of non-stress cells and also BHA-treated cells (Figure 4.1- Figure 4.3). Santoro and Thiele (1997) reported that *GSH1* and *TRX2* genes are controlled by Yap1 transcription factor. The *GSH1* was found to be induced by both H_2O_2 and menadione whereas the *TRX2* gene was induced by H_2O_2 (Stephen, et al., 1995; Stephen and Jamieson, 1996). When cells were pre-treated with low concentrations of BHA and then subsequently exposed to H_2O_2, it was noticed that the expression of *TRX2-lacZ*, *SSA1-lacZ* and *GSH1-lacZ* genes was higher than non-stressed and BHA-treated cells, however some parameters displayed lower significant difference in expression levels compared to H_2O_2 treatment alone (Figure 4.1- Figure 4.3). These results indicate that BHA does not function by inducing expression of these genes and elevated activities and levels of their gene products are not responsible for the observed BHA mediated H_2O_2 cross protection.

6.3 A genetic approach to identifying genes involved in BHA sensitivity

Mutants which were sensitive to BHA were generated by chemical mutagenesis to identify genes which are responsible for BHA toxicity. BHA sensitive mutants were isolated and complementation tests were carried out to determine the number of genes defined by the mutant. The data showed that the mutations were recessive and defined at least 6 genes (Figure 5.1). Therefore, to isolate and identify genes defined by the mutants, cloning by complementation was performed. Nasmyth and Reed (1980) suggested that this method could be applicable to any yeast gene for which mutant phenotype is scorable.

For these experiments I chose yeast genomic DNA libraries constructed in YEp13 and YEp24 as the plasmids copy number is high, at about 20-50 per cell (Parent and Bostian, 1995; Brown, 2010). This would also allow for the isolation of copy number suppressors in addition to clones that complement the mutant alleles directly. Although several transformations were done, only 8 transformants complemented the BHA sensitivity phenotype (Table 5.4 and Table 5.5). Many of the Leu^+ and Ura^+ transformants failed to grow on YPD containing BHA, possibly because of plasmids are instable. Additionally, Ausubel, et al. (2003) reported that some yeast genes could be toxic when expressed in *E.coli*, resulting in preexisting yeast libraries which had been amplified in *E.coli* might be under-represented in plasmids containing these genes.

Once the plasmids were isolated from these transformants, the purified plasmids were re-transformed back into their original mutants to prove that these plasmids responsible for complementation of BHA sensitive mutation. There was one re-transformant which contained plasmid pYCC7, which was unable to complement the BHA sensitive mutation. Possibly there might be more than one plasmid in the original transformant (Ausubel, et al., 2003) or alternatively it might be that instability of the plasmid or the original BHA sensitive mutation has reverted. In the case of reversion of original mutant, suppressor analysis might be an alternative approach to identify gene if the time of experimentation had no limit.

6.4 The effect of plasmids on the BHA sensitive phenotype

Regarding to the re-transformant phenotype, in this study showed that the phenotype of some re-transformants was unstable. The MS-50 re-transformant carrying purified plasmid pYCC6 which initially complemented the BHA sensitive phenotype was found to no longer complement it after repeat spot testing. It is probable that the plasmid

might be instable. Futcher and Cox (1984) reported that instability of plasmid is negatively correlated to plasmid copy number. Higher instability was found on random partition of the plasmid. Furthermore, Zhang, et al. (1996) reported that unstable plasmid tend to lose the transformant properties. Plasmid instability possibly caused by deletion, insertion, recombination or other events at the level of the DNA which is known as structural instability. In addition to structural instability, the instability of plasmid may be caused by uneven partitioning of plasmids during cell division (Futcher and Cox, 1984; Armstrong, et al., 1989; Zhang, et al., 1996). These findings might support my findings that the yeast transformants carrying either YEp13 or pYCC3 (high copy number of 2 μm circle based plasmid containing *LEU2*) conferred a BHA resistance phenotype on YPD+0.8 mM BHA. Whereas yeast transformants carrying pRS415 which is low copy number plasmid (*CEN-ARS LEU2*) did not have the ability to grow on YPD+0.8 mM BHA (Table 5.7). Additionally, it was noticed that the BHA resistance phenotype varied depending upon the mutants. For instance, the mutants MS-10 and MS-24 which are in different complementation groups exhibited different BHA resistance phenotype of their transformants (Section 5.4). This possibly indicates that the ability of alleles in different genes to be suppressed by the presence of a high copy number plasmid may affect BHA resistance.

6.5 Genes involved in BHA toxicity

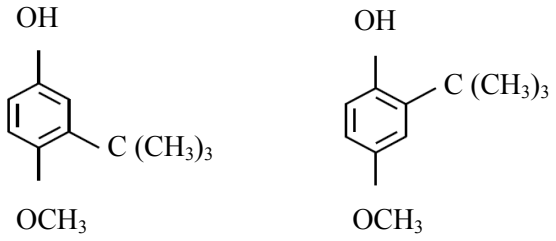
As the results shown in section 5.2, reveal there are 7 candidate plasmids (pYCC1, pYCC2, pYCC3, pYCC4, pYCC5, pYCC6 and pYQZ) which complement the BHA sensitivity phenotype of the transformants. These plasmids were sequenced to identify the genes that respond to the BHA sensitivity. The data show there are 3 genes (*TYR1*, *KRE6* and *GPH1*) possibly involved with BHA toxicity. The first is *TYR1*, the gene from *S. cerevisiae* represents in a region of chromosome II. This gene encodes prephenate dehydrogenase which is involved in tyrosine biosynthesis (Mannhaupt, et al.,1989; *Saccharomyces* Genome Database). The second gene is *KRE6*, encoding type II membrane protein required in β-1,6 glucan biosynthesis (Roemer and Bussey, 1991; *Saccharomyces* Genome Database). The third gene is *GPH1*, encoding glycogen phosphorylase. *GPH1* expression is regulated by stress-response elements and by the HOG MAP kinase pathway. *GPH1* is located near the gene *KRE6* on chromosome XVI of *S. cerevisiae* (Sunnarborg, et al., 2001; *Saccharomyces* Genome Database).

The *TYR1* gene was identified in plasmid pYCC1 which came from transformant of the MB2-36 mutant. This complemented the BHA sensitive phenotype of the MB2-36 mutant. However, the yeast strain lacking the *TYR1* gene had an identical phenotype to the MB2-36 (Figure 5.4). In addition, the MB2-36 mutant was found to be auxotrophic for tyrosine. As a result it seems to be evident that the Tyr1 enzyme is at least partially responsible for countering BHA toxicity.

Interestingly, Lupo, et al. (1997) reported that an *S. cerevisiae* mutant with impaired tyrosine synthesis was sensitive to H_2O_2. The research suggested that tyrosine could participate in protection against oxidative stress in *S. cerevisiae*. There were two possible pathways in which tyrosine could be involved in protection against oxidative stress. One pathway is the formation of dityrosine, leading to the decomposition of H_2O_2. This pathway was observed in human neutrophils and macrophages. Dityrosine can be synthesized by myeloperoxidase-H_2O_2 system, the enzyme myeloperoxidase is secreted by activated phagocytes, and requires H_2O_2 to oxidize L-tyrosine to yield dityrosine (Heinecke, et al., 1993). It could be implied that tyrosine might be involved in protecting cell against oxidative stress by the peroxidase pathway. Another possibility to explain a role of tyrosine in oxidative stress is that the phenolic group may be able to scavenge oxygen free radicals (Lupo, et al., 1997). Rice-Evans, et al. (1997) reported that phenolic compounds could act as hydrogen or electron-donating agents, lead to scavenging of free-radical. In addition Gülçin (2007) revealed that L-tyrosine, a monophenolic amino acid and L-Dopa, a diphenolic amino acid exhibited antioxidant activities in different assays, including superoxide radical scavenging, hydrogen peroxide scavenging, antiradical activities, total ferric ions reducing power and metal chelating activities.

In the case of this study, although BHA might not directly function by inducing classical oxidant genes to protect against oxidative stress, BHA still exhibited antioxidant properties. This might be due to BHA inducing *TYR1* gene expression in yeast cells resulting in increased levels of tyrosine which could scavenge oxygen free radicals, this should be studied further. For tyrosine biosynthesis in the yeast *S. cerevisiae*, the enzyme prephenate dehydrogenase, which is encoded by the *TYR1* gene, catalyses prephenate to 4-hydroxyphenylpyruvate by the oxidative decarboxylation and dehydratation. Then, 4-hydroxyphenylpyruvate is converted to tyrosine by the enzyme aromatic amino acid aminotransferase (Braus, 1991). The possibility that correlation between BHA and *TYR1* gene is the structure of BHA might look like the substrate of

the enzyme encoded by the *TYR1* gene, as a result in Tyr1 enzyme (prephenate dehydrogenase) might catalyze BHA. The BHA structure and tyrosine biosynthesis pathway are illustrated in Figure 6.1 and Figure 6.2.



3-isomer 2-isomer

Source: Berdahl, et al. (2010)

Figure 6.1 Butylated hydroxyanisole (BHA) structures

In addition to the *TYR1* gene, *KRE6* and *GPH1* may participate in the BHA stress response. These genes were identified from the plasmid pYCC5 which were isolated from the transformant of MS-54. A number of studies reported that *KRE6*, encoding type II membrane protein involved in β-glucan synthesis of yeast cell wall. Roemer and Bussey (1991) demonstrated that disruption of *KRE6* gene in the yeast cells led to the mutants grew up slowly, cells were abnormal large and levels of both β-1,3-glucan and β-1,6-glucan synthase were reduce. In addition, *SKN1*, encodes a protein sharing 66% identity to Kre6p was found to function independently in β-1,6-glucan synthesis (Roemer, et al., 1993). Roemer, et al. (1994) also found that protein kinase C, *PKC1* pathway participates with Kre6p and Skn1p in response to (1→6)-β-glucan synthesis. Deletion of *PKC1* gene in *S. cerevisiae* exhibits cell lysis defect. Roemer, et al. (1994) showed overexpression of *KRE6* can alleviate *pkc1* lysis defect, implying that *KRE6* and *PKC1* have interaction in cell wall synthesis. When the yeast cell wall was perturbed by general stresses such as chemical drugs, osmotic shock and temperature, a repair mechanism can be triggered. Lagorce, et al. (2003) investigated cell wall repair mechanism by comparison of global gene expression in the *S. cerevisae* mutants; *fks1*, *kre6*, *mnn9*, *gas1*, and *knr4*. They found that this mechanism may involve in three regulatory systems; the *PKC1*-*SLT2* MAPK signal module, the general stress response

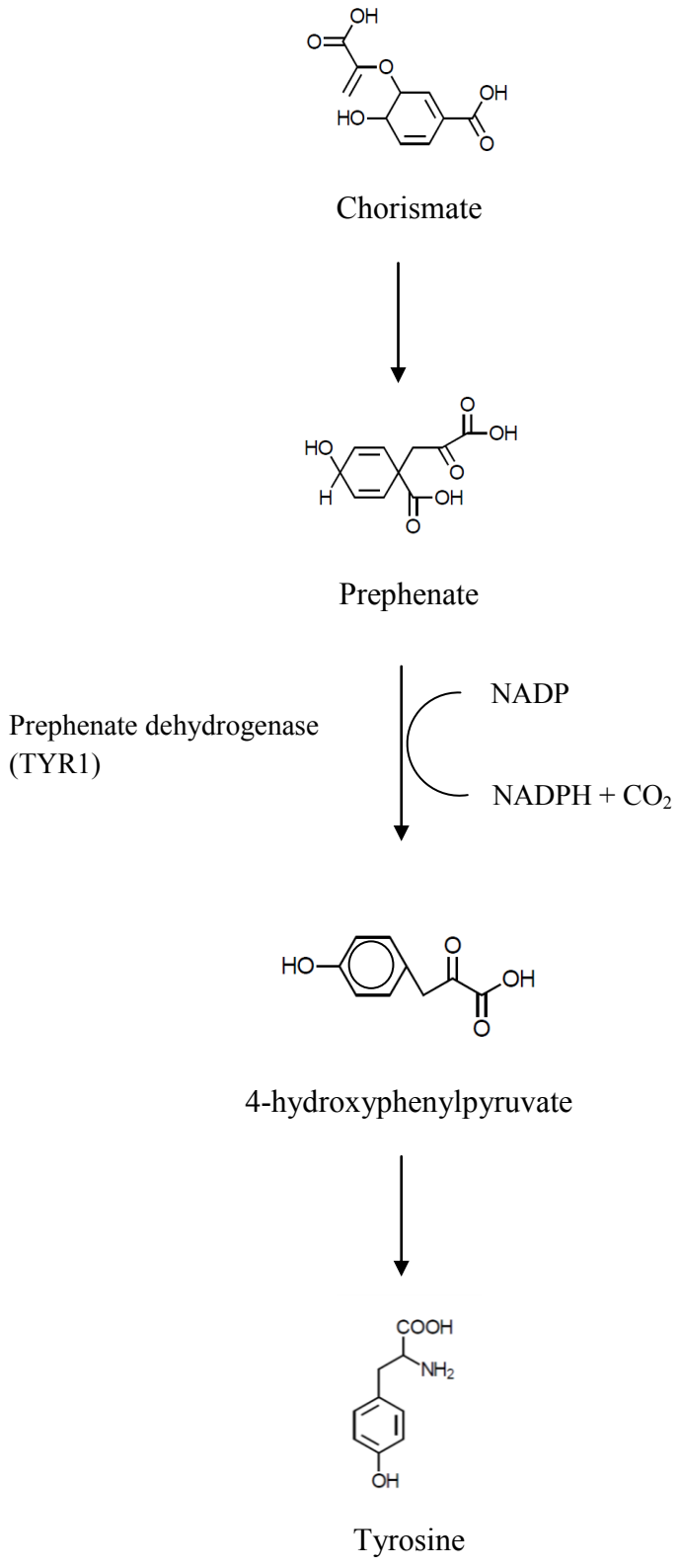
mediated by Msn2/4p and the Ca^{2+}/calcineurin-dependent signaling pathway. In view of BHA stress response, it is possible that BHA treatment might perturb the yeast cell wall, resulting in cell wall proteins might be induced to repair cell wall damage. However, the correlation between *KRE6* and BHA is not clear.

In turn *GPH1*, Sunnarborg, et al. (2001) reported that the expression of *GPH1* is regulated by stress response elements (STREs) which are responsible for heat shock, osmotic shock and growth in stationary phase. Furthermore, Favre, et al. (2008) revealed that the lack of *GPH1* led to increasing the level of ROS, decreasing the expression of superoxide dismutase genes and shorting the life span of yeast. This study suggested that *GPH1* might participate in metabolic changes due to other stress responses of yeast cells.

Although the results found that three genes; *TYR1*, *KRE6* and *GPH1* were responsible for BHA toxicity, the relationship between these genes and BHA is still unclear. To gain a better understanding of the mechanism of action of BHA in yeast cells, sub-cloning of these genes, phenotypic spot testing and determining the levels of enzymes such as catalase, superoxide dismutase and glutathione peroxidase should further be studied. Additionally, microarray analysis should be carried out in order to study gene expressions during BHA stress response.

6.6 Conclusion

In summary, the results of this study indicate that pre-treatment of yeast cells with low concentrations of BHA can induce protection towards toxic levels of both BHA and H_2O_2. However, the data show that *S. cerevisiae* S150-2B (wild type) is more sensitive to BHA than yeast strain lacking Yap1, a key regulator of oxidative stress. Also, treatment of the *S. cerevisiae* with various levels of BHA cannot induce the expression of oxidant-regulated genes (*TRX2* and *GSH1*). It seems BHA could not act through an oxidative route. In addition, the *TYR1*, *KRE6* and *GPH1* genes were found to be involved in countering BHA toxicity in *S. cerevisiae*.



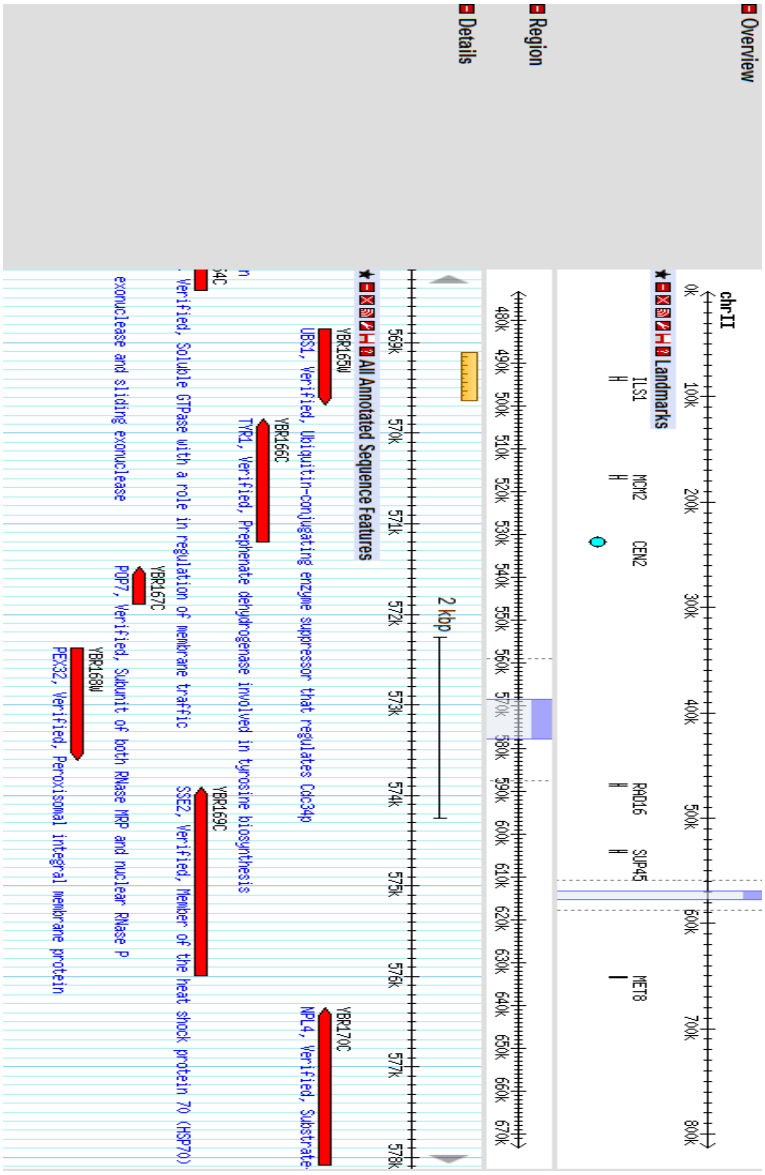
Source: Braus (1991) and *Saccharomyces cerevisiae* Pathway: tyrosine biosynthesis (http://pathway.yeastgenome.org)

Figure 6.2 The tyrosine biosysthesis pathway

APPENDIX

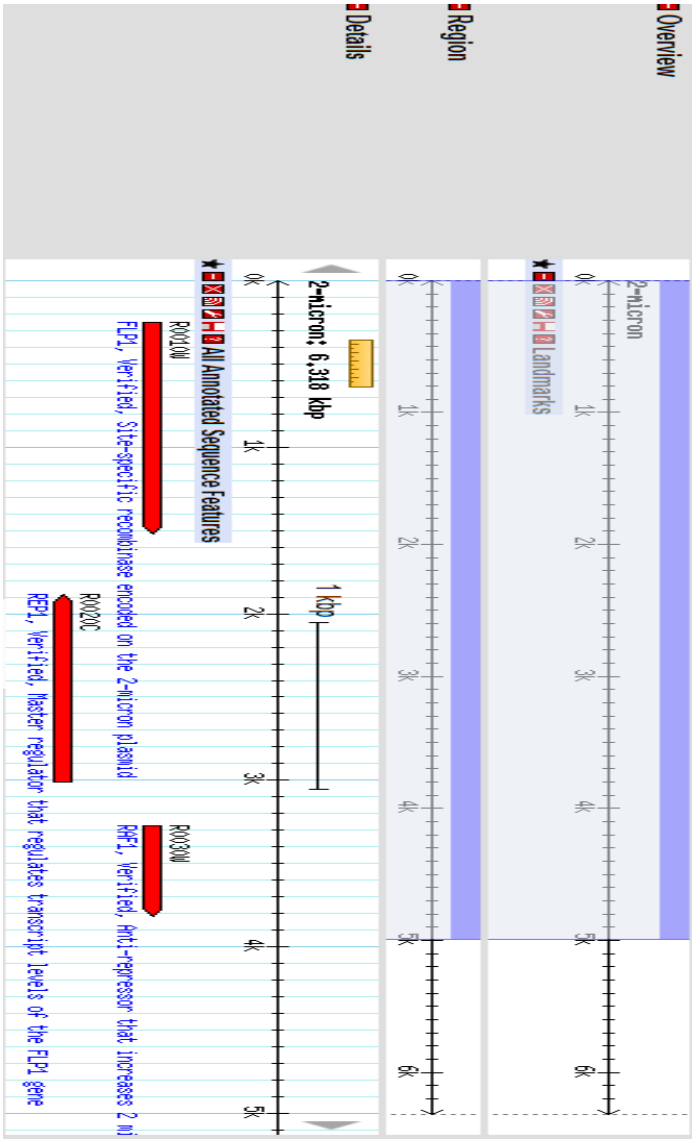
Appendix A. Maps of genomic DNA fragment in the complementing plasmid pYCC1, pYCC3, pYCC5, pYCC6 and pYQZ

The DNA insert of each complementing plasmid was sequenced and compared with *Saccharomyces* genome database (SGD) by performing BLAST searches. The genomic maps of DNA insert of each complementing plasmids are shown below.



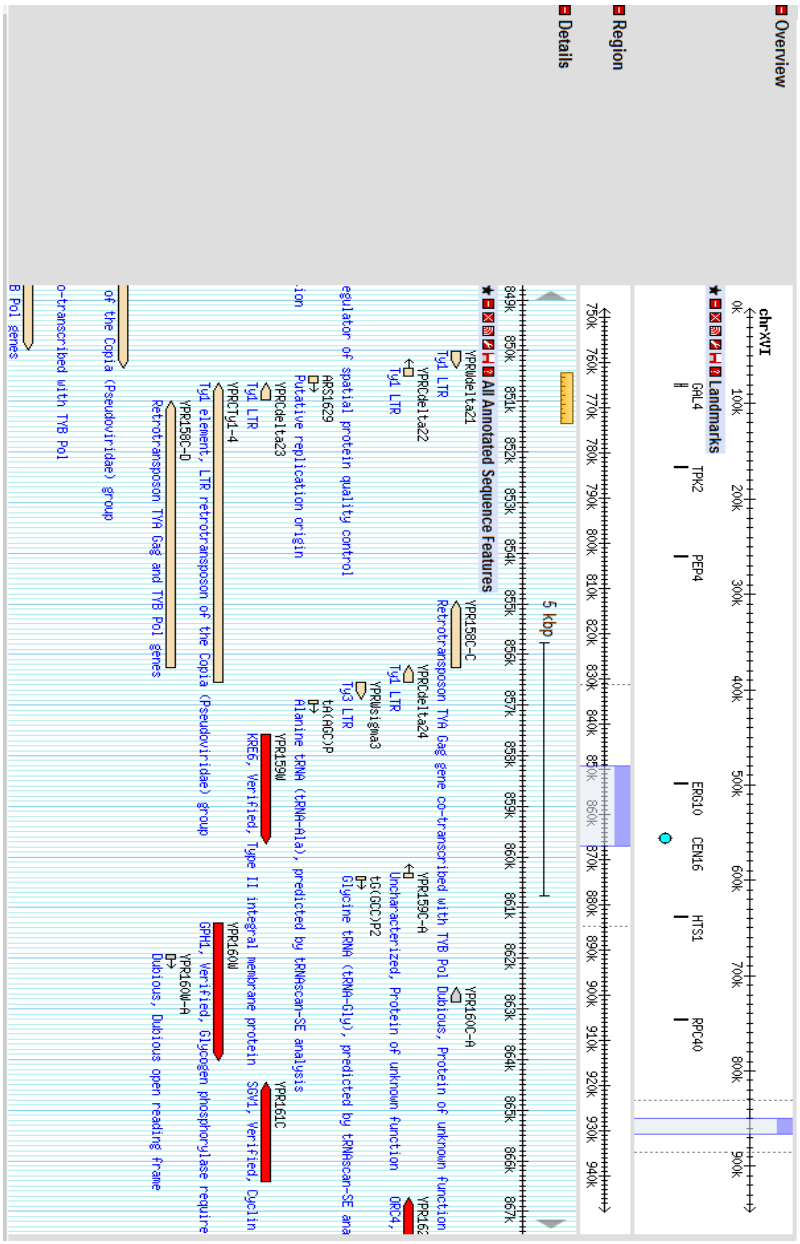
Source: www.yeastgenome.org

Figure 7.1 The genomic map of the DNA insert in plasmid pYCC1. The boundaries of the insert DNA is from 568433 bp to 577895 bp of Chromosome II.



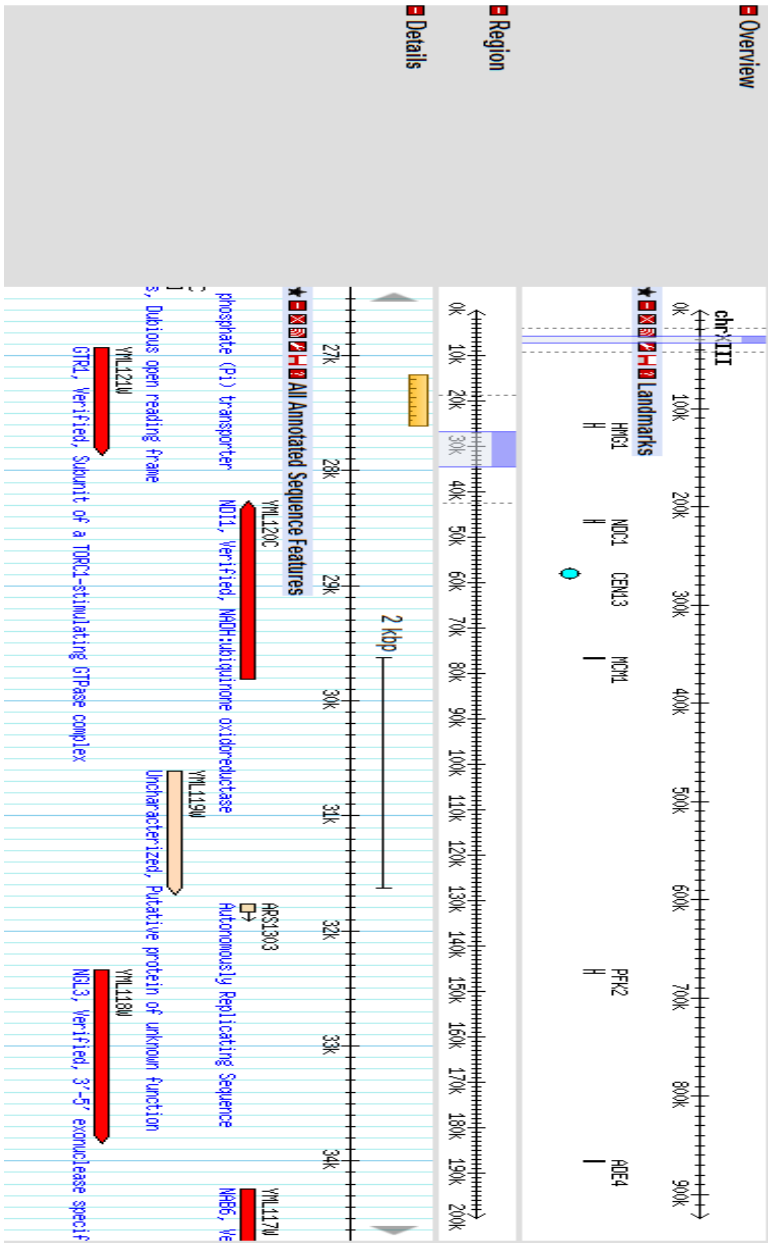
Source: www.yeastgenome.org

Figure 7.2 The genomic map of the DNA insert in plasmid pYCC3. The boundaries of the insert DNA is from 1 bp to 5000 bp of the 2-micron yeast plasmid.



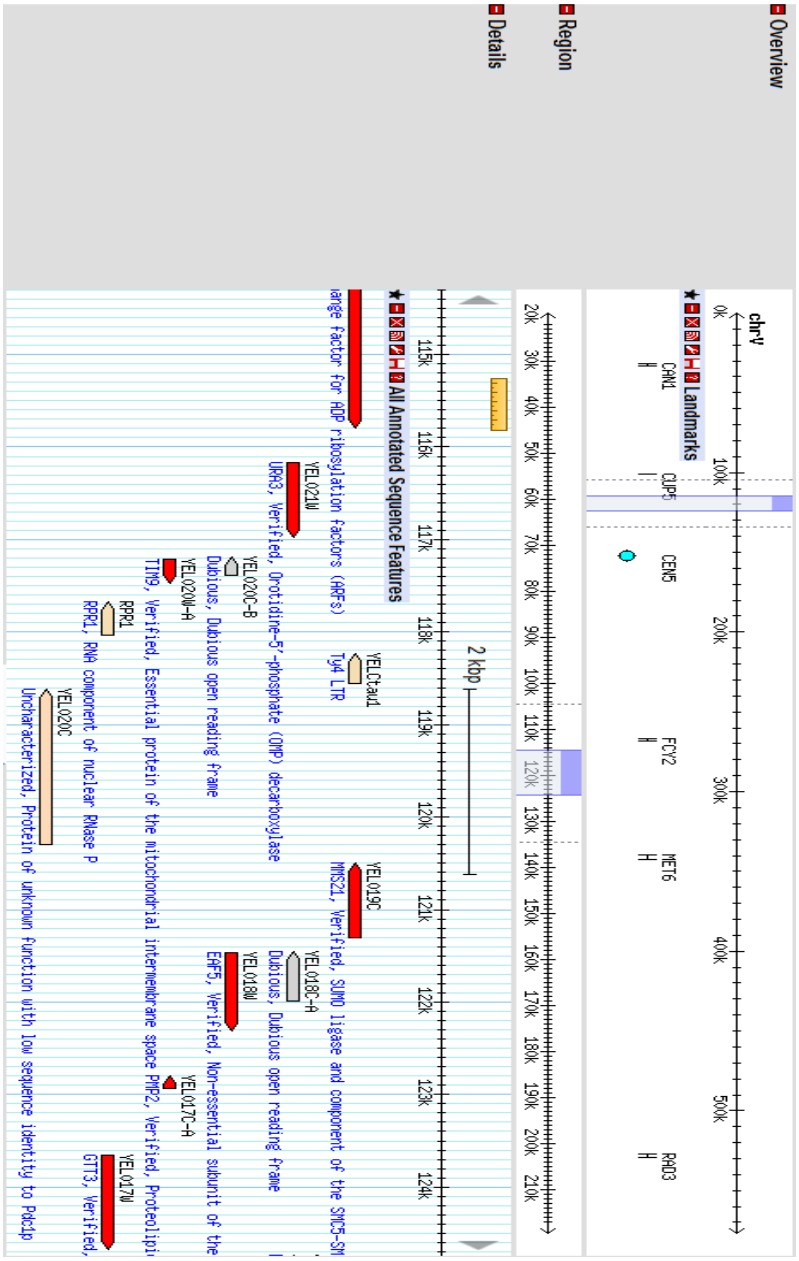
Source: www.yeastgenome.org

Figure 7.3 The genomic map of the DNA insert in plasmid pYCC5. The boundaries of the insert DNA is from 849164 bp to 867014 bp on Chromosome XVI.



Source: www.yeastgenome.org

Figure 7.4 The genomic map of the DNA insert in plasmid pYCC6. The boundaries of the insert DNA is from 26603 bp to 34500 bp on Chromosome XIII.



Source: www.yeastgenome.org

Figure 7.5 The genomic map of the DNA insert in plasmid pYQZ. The boundaries of the insert DNA is from 114551 bp to 124550 bp on Chromosome V.

Appendix B. Analysis of digested plasmids

The complementing plasmid pYCC1, pYCC2, pYCC3, pYCC4, pYCC5, pYCC6 and pYQZ were analyzed by agarose gel electrophoresis. Reaction of plasmid digestion was described in section 2.12. Visualization of these digested plasmids is shown in figure 7.6 and 7.7.

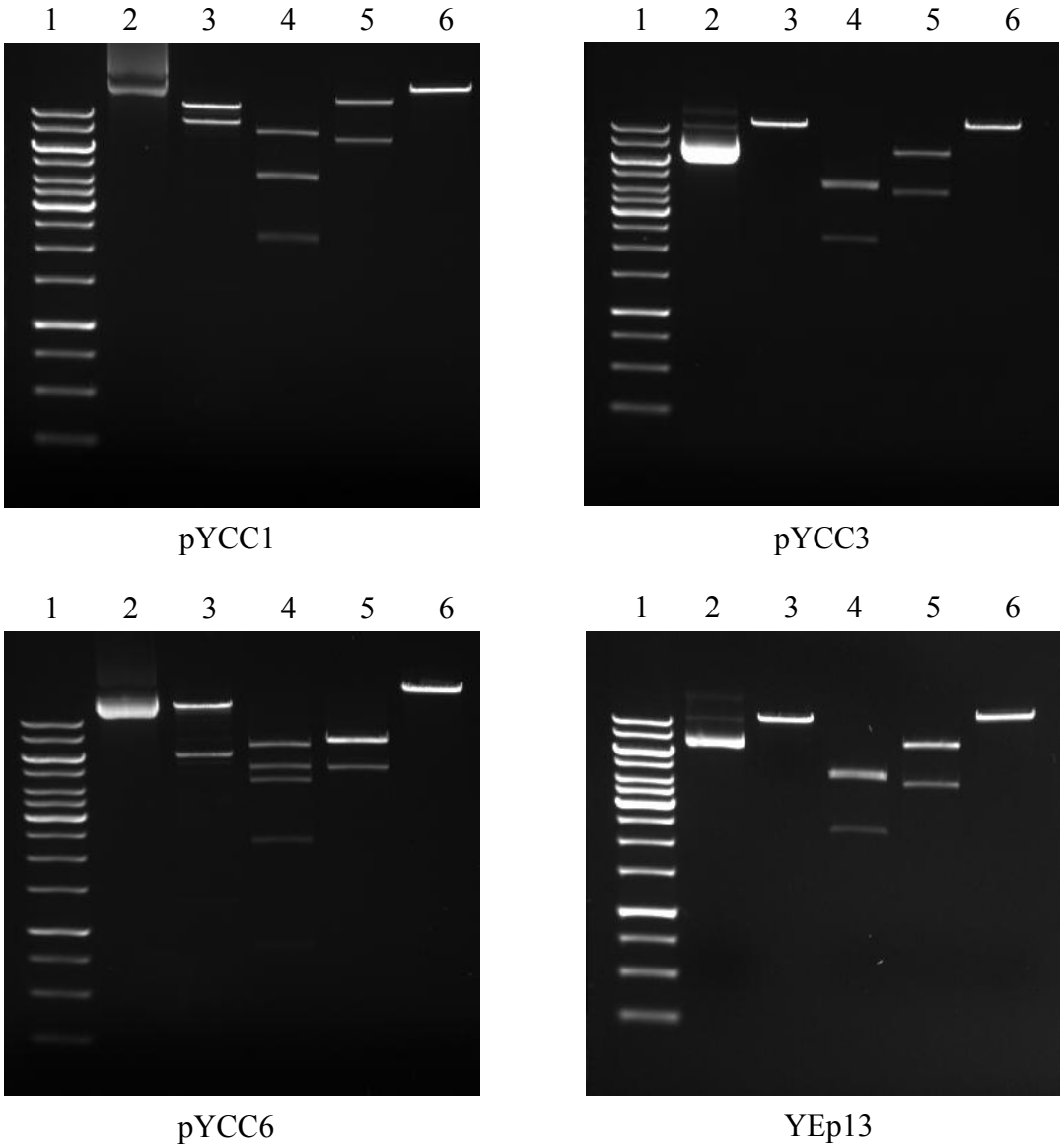
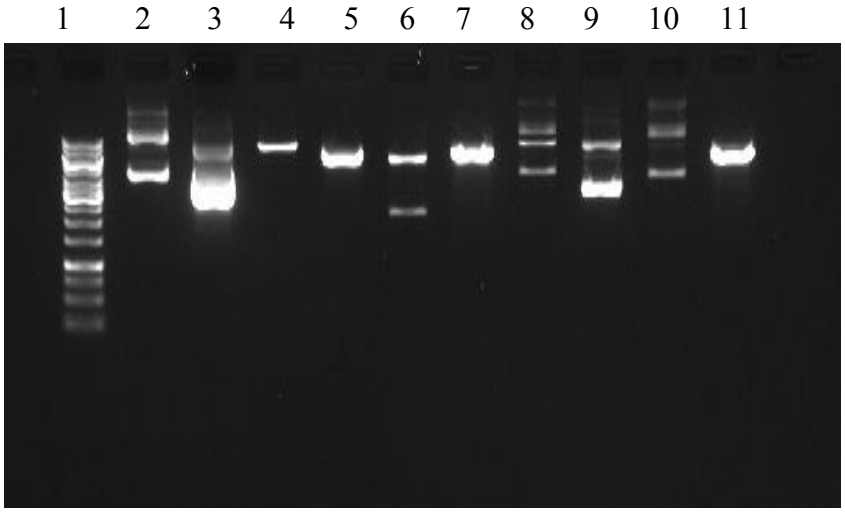
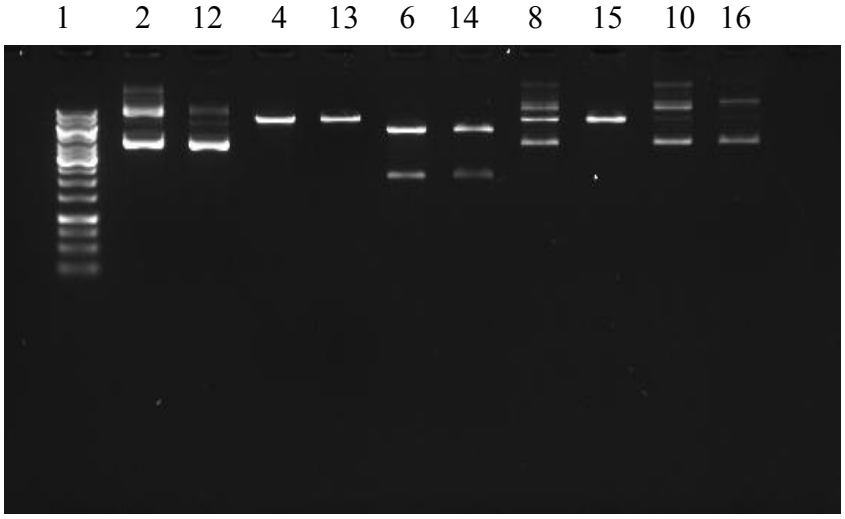


Figure 7.6 Example of visualizing digested plasmid pYCC1, pYCC3, pYCC6 and YEp13 (plasmid background) in agarose gel. Plasmid DNA was electrophoresed in 0.8% agarose gel for 45 minutes at 45 mV. The positions in the gel represent 1kb marker DNA (1), circular DNA (2), digested plasmid DNA with *Bam*HI (3), *Eco*RI (4), *Sal*I (5) and *Xho*I (6).



pYCC5



pYQZ

Figure 7.7 Visualizing digested plasmid pYCC5, pYQZ and YEp24 (plasmid background) in agarose gel. Plasmid DNA was electrophoresed in 0.8% agarose gel for 45 minutes at 45 mV. The positions in the gel represent 1kb marker DNA (1), circular DNA YEp24(2), circular DNA pYCC5 (3), digested YEp24 with *Bam*HI (4), digested pYCC5 with *Bam*HI (5), digested YEp24 with *Eco*RI (6), digested pYCC5 with *Eco*RI (7), digested YEp24 with *Sal*I (8), digested pYCC5 with *Sal*I (9), digested YEp24 with *Xho*I (10), digested pYCC5 with *Xho*I (11), circular DNA pYQZ (12), digested pYQZ with *Bam*HI (13), digested pYQZ with *Eco*RI (14), digested pYQZ with *Sal*I (15) and digested pYQZ with *Xho*I (16).

REFERENCES

Ali Ramadan, A.M. and Suzuki, T. (2012) Detection of genotoxicity of phenolic antioxidants, butylated hydroxyanisole and tert-butylhydroquinone in multiple mouse organs by the alkaline comet assay. *Journal of American Science*, 8(1), 722-727.

Allen, R.G., and Tresini, M. (2000) Oxidative stress and gene regulation. *Free radical biology & Medicine*, 28(3), 463-499.

Amorós, M. and Estruch, F. (2001) Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes *HSP26* and *HSP104* in a gene- and stress type-dependent manner. *Molecular Microbiology*, 39(6), 1523-1532.

Anonymous (2011) Report on carcinogen, Twelfth edition. Butylated hydroxyanisole. National toxicology program, Department of Health and Human Services, 78-79.

Armstrong, K.A., Som, T., Volkert, F.C., Rose, A. and Broach, J.R. (1989) Propagation and expression of genes in yeast using 2-micron circle vectors. In Barr, P.J., Brake, A.J. and Valenzuela, P. (eds.). *Yeast genetic engineering*. USA: Butterworth publishers, 165-192.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (2003) Cloning yeast genes by complementation. In *Current Protocols in Molecular Biology*, USA: Jonh Wiley & Sons, Inc, 13.8.1-13.8.4.

Ball, S.G., Wickner, R.B., Cottarel, G., Schaus, M. And Tirtiaux, C. (1986) Molecular cloning and characterization of *ARO7-OSM2*, a single yeast gene necessary for chorismate mutase activity and growth in hypertonic medium. *Molecular and General Genetics MGG*, 205, 326-330.

Bednarska, S., Leroy, P., Zagulski, M. and Bartosz, G. (2008) Efficacy of antioxidants in the yeast *Saccharomyces cerevisiae* correlated with their effects on protein thiols. *Biochimie*, 90, 1476-1485.

Berdahl, D.R., Nahas, R.I., Barren, J.P. (2010) Synthetic and natural antioxidant additives in food stabilization: current applications and future research. In Decker, E.A., Elias, R.J. and McMlements, D.J. (eds.). *Oxidation in foods and beverages and antioxidant applications Volume 1: Understanding mechanisms of oxidation and antioxidant activity*. Cambridge: Woodhead Publishing Limited, 272-320.

Berry, D.B. and Gasch, A.P. (2008) Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Molecular Biology of the cell*, 19, 4580-4587.

Biryukova, E.N., Medentsev, A.G., Arinbasarova, A.Y. and Akimenko, V.K. (2008) Respiratory activity of yeast *Yarrowia lipolytica* under oxidative stress and heat shock. *Microbiology*, 77(4), 395-399.

Braus, G.H. (1991) Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*: a model system for the regulation of a eukaryotic biosynthesis pathway. *Microbiological Reviews*, 55(3), 349-370.

Brown, T.A. (2010) Cloning vectors for eukaryotes. In *Gene cloning and DNA analysis: An introduction*, 6th ed., UK: Wiley-Blackwell, 105-125.

Cadenas, E. and Davies, K.J.A. (2000) Mitochondrial free radical generation, oxidative stress, and aging. *Free Radical Biology & Medicine*, 29(3/4), 222-230.

Carmel-Harel, O. and Storz, G. (2000) Roles of the glutathione- and thioredoxin-dependent reduction systems in the *Escherichia coli* and *Saccharomyces cerevisiae* responses to oxidative stress. *Annu. Rev. Microbiol*, 54, 439–61.

Carmel-Harel, O., Stearman, R., Gasch, A.P, Botstein, D., Brown, P.O. and Storz, G. (2001) Role of thioredoxin reductase in the Yap1p-dependent response to oxidative stress in *Saccharomyces cerevisiae*. *Molecular Microbiology*, 39, 595-605.

Choe, E. and Min, D.B. (2009) Mechanisms of antioxidants in the oxidation of foods. *Comprehensive reviews in Food Science and Food Safety*, 8, 345-358.

CODEX. (2011) GSFA online food additive index [online]. Available from: http://www.codexalimentarius.net/gsfaonline/additives/index.html [accessed 16 March 2012].

Collinson, L.P. and Dawes, I.W. (1992) Inducibility of the response of yeast cells to peroxide stress. *Journal of general Microbiology*, 138, 329-335.

Estruch, F. (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. *FEMS Microbiology Reviews*, 24, 469-486.

Favre, C., Aguilar, P.S. and Carrillo, M.C. (2008) Oxidative stress and chronological aging in glycogen-phosphorylase-deleted yeast. *Free Radical Biology & Medicine*, 45, 1446-1456.

Flattery-O'Brien, J., Collinson, L.P. and Dawes, I.W. (1993) *Saccharomyces cerevisiae* has an inducible response to menadione which differs from that to hydrogen peroxide. *Journal of General Microbiology*, 139, 501-507.

Futcher, A.B. and Cox, B.S. (1984) Copy number and the stability of 2-μm circle-based artificial plasmids of *Saccharomyces cerevisiae*. *Journal of Bacteriology*, 157 (1), 283-290.

Garrido, E.O. and Grant, C.M. (2002) Role of thioredoxins in the response of *Saccharomyces cerevisiae* to oxidative stress induced by hydrogen peroxides. *Molecular Microbiology*, 43(4), 993-1003.

Gasch, A.P., Spellman, P.I., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., Botstein, D. and Brown, P.O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Molecular Biology of the Cell*, 11, 4241-4257.

Gasch, A.P. (2010) The environmental stress response: a common yeast response to diverse environmental stresses. In Hohmann, S. and Mager, W.H. (eds.). *Topics in current genetics1: Yeast stress responses*. Germany: Springer-Verlag Berlin Heidelberg, 11-70.

Gietz, R.D. and Woods, R.A. (2002) Transformation of yeast by the Liac/ss carrier DNA/ PEG method. *Methods in Enzymology*, 350, 87-96.

Grant, C.M., MacIver, F.H. and Dawes, I.W. (1997) Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide y-glutamylcysteine, *Molecular Biology of the Cell*, 8, 1699-1707.

Grant, C.M., Perrone, G. and Dawes, I.W. (1998) Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast *Saccharomyces cerevisie*. *Biochemical and Biophysical Research Communications*, 253, 893-898.

Grant, C.M. (2001) Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress conditions. *Molecular Microbiology*, 39(3), 533-541.

Greetham, D. and Grant, C.M. (2009) Antioxidant activity of the yeast mitochondrial one-cys peroxiredoxin is dependent on thioredoxin reductase and glutathione in vivo. *Molecular and cellular biology*, 29(11), 3229-3240.

Guidot, D.M., McCord, J.M., Wright, R.M. and Repine, J.E. (1993) Absence of electron transport (Rho 0 state) restores growth of a manganese-superoxide dismutase deficient *Saccharomyces cerevisiae* in hyperoxia. Evidence for electron transport as a major source of superoxide generation *in vivo*. *The Journal of Biological Chemistry*, 268, 26699-26703.

Gülçin, İ. (2007) Comparison of in vitro antioxidant and antiradical activities of L-tyrosine and L-Dopa. *Amino Acids*, 32, 431-438.

Halliwell, B., Aeschbach, R., Löliger, J. and Aruoma, O.I. (1995) The characterization of antioxidants. *Fd Chem*. *Toxic*., 33(7), 601-617.

Heinecke, J.W., Li, W., Daehnke III, H.L. and Goldstein, J.A. (1993) Dityrosine, a Specific Marker of Oxidation, Is Synthesized by the Myeloperoxidase-Hydrogen Peroxide System of Human Neutrophils and Macrophages. *The journal of Biological Chemistry*, 268(6), 4069-4077.

Herrero, E., Ros, J., Belli, G. and Cabiscol, E. (2008) Redox control and oxidative stress in yeast cells. *Biochimica et Biophysica Acta*, 1780, 1217-1235.

Hohmann, S. and Mager, W.H. (2010) Introduction. In Hohmann, S. and Mager, W.H. (eds.). *Topics in current genetics 1 Yeast stress responses*. Germany: Springer-Verlag Berlin Heidelberg, 1-9.

IARC. (1986) Butylated hydroxyanisole (BHA). *International Agency for Research on Cancer (IARC) Summaries & evaluations* [online]. Available from: http://www.inchem.org/documents/iarc/vol40/butylatedhydroxyanisole.html [accessed 16 March 2012].

Izawa, S., Inoue, Y. And Kimura, A. (1995) Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*. *FEBS Letters*, 368, 73-76.

Jamieson, D.J. (1992) *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. *Journal of Bacteriology*, 174, 6678-6681.

Jamieson, D.J., Rivers, S.L. and Stephen, D.W.S. (1994) Analysis of *Saccharomyces cerevisiae* proteins induced by peroxide and superoxide stress. *Microbiology*, 140, 3277-3283.

Jamieson, D.J., Stephen, D.W.S. and Terriere, E.C. (1996) Analysis of the adaptive oxidative stress response of *Candida albicans*. *FEMS Microbiology Letters*, 138, 83-88.

Jamieson, D.J. (1998) Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast*, 14, 1511-1527.

Kahl, R. (1984) Synthetic antioxidants: biochemical actions and interferences with radiation and toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology*, 33, 185-228.

Kahl, R., Weinke, S. and Kappus, H. (1989) Production of reactive oxygen species due to metabolic activation of butylated hydroxyanisole. *Toxicology*, 59, 179-194.

Kehrer, J.P (2000) The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology*, 149, 43-50.

Kuge, S. and Jones, N. (1994) YAP1 dependent activation of TRX2 is essential for the response of Saccharomyces cerevisiae to oxidative stress by hydroperoxides. *The EMBO Journal*, 13(3), 655-664.

Lagorce, A., Hauser, N.C., Labourdette, D., Rodriguez, C., Martin-Yken, H., Arroyo, J., Hoheisel, J.D. and François, J. (2003) Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae*. *The Journal of Biological Chemistry*, 278(22), 20345-20357.

Larson, R.A. (1995) Antioxidant mechanisms of secondary natural product. In Ahmad, S. (ed.). *Oxidative stress and antioxidant defense in Biology*. New York: Chapman & Hall, 210-233.

Lawrence, C.W. (2002) Classical Mutagenesis Techniques. In Guthrie, C. and Fink, G.R. (eds.). *Guide to yeast genetics and molecular and cell biology part B, Methods in Enzymology vol. 350*. San Diego: Academic Press, 189-198.

Lee, J., Godon, C., Lagnie, G., Spector, D., Garin, J., Labarre, J. And Toledano, M.B. (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *The Journal of Biological Chemistry*, 274, 16040-16046.

Lu, F., Wang, Y., Bai, D. and Du, L. (2005) Adaptive response of *Saccharomyces cerevisiae* to hyperosmotic and oxidative stress. *Process Biochemistry*, 40, 3614-3618.

Lupo, S., Aranda, C., Miranda-Ham, L., Olivera, H., Riego, L., Servin, L. and Gonzalez, A. (1997) Tyrosine is involved in protection from oxidative stress in *Saccharomyces cerevisiae*. *Can. J. Microbiol*, 43, 963-970.

Mager, W.H. and Hohmann, S. (1997) Introduction: Stress response mechanisms in yeast *Saccharomyces cerevisiae*. In Hohmann, S. and Mager, W.H. (eds.). *Molecular Biology Intelligence unit: Yeast stress responses*. Texas: R.G Landes company, 1-6.

Mannhaupt, G., Stucka, R., Pilz, U., Schwarzlose, C. and Feldmann, H. (1989) Characterization of the prephenate dehydrogenase-encoding gene, TYR1, from *Saccharomyces cerevisiae*. *Gene,* 85(2), 303-11.

Mastinez-Pastor, M.T., Marchler, G., Schüller, C., Marchler-Bauer, A., Ruis, H. and Estruch, F. (1996) The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element (STRE). *The EMBO Journal*, 15(9), 2227-2235.

Masutani, H., Ueno, M., Ueda, S. and Yodoi, J. (2000) Role of thioredoxin and redox regulation in oxidative stress response and signaling. In Sen, C.K., Sies, H. and Baeuerle, P.A. (eds.). *Antioxidant and redox regulation of genes*. San Diego, California: Academic Press, 297-310.

Matés, J.M. (2000) Effects of antioxidant enzymes in the molecular control of reactive oxygen species toxicology. *Toxicology*, 153, 83-104.

Moon, H.J. and Park, W.H. (2011) Butylated hydroxyanisole inhibits the growth of HeLa cervical cancer cells via caspase-dependent apoptosis and GSH depletion. *Mol.Cell.Biochem*, 344, 179-186.

Moradas-Ferreira, P. and Costa, V. (2000) Adaptive response of the yeast *Saccharomyces cerevisiae* to reactive oxygen species: defences, damage and death. *Redox Report*, 5(5), 277-285.

Morano, K.A., Grant, C.M. and Moye-Rowley, W.S. (2012) The response to heat shock and oxidative stress in *Saccharomyces cerevisiae*. *Genetics*, 190, 1157-1195.

Morgan, B.A., Banks, G. R., Toone, W.M., Raitt, D., Kuge, S. and Johnson, L.H. (1997) The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cervisiae*. *The EMBO Journal*, 16(5), 1035-1044.

Murphy, M. P. (2009) How mitochondria produce reactive oxygen species. *Biochem. J.*, 417, 1-13.

Nasmyth, K.A. and Reed, S.I. (1980) Isolation of genes by complementation in yeast: Molecular cloning of a cell-cycle gene. *Proc. Natl. Acad. Sci. USA*, 77(4), 2119-2123.

Nimse, S. B. and Pal, D. (2015) Free radicals, natural antioxidants, and their reaction mechanism. *RSC Advances*, 5, 27986-28006.

Ouyang, X., Tran, Q.T., Goodwin, S.,Wible, R.S., Sutter, C.H. and Sutter, T.R. (2011) Yap1 activation by H_2O_2 or thiol-reactive chemicals elicits distinct adaptive gene response. *Free Radical Biology & Medicine*, 50, 11-13.

Parent, S.A. and Bostian, K.A. (1995) Recombinant DNA technology: yeast vectors. In Rose, A.H., Wheals, A.E. and Harrison, J.S. (eds.). *The Yeasts volume 6: Yeast Genetics*. London: Academic Press, 121-178.

Porter, A.G. and Jänicke, R.U. (1999) Emerging roles of caspase-3 in apoptosis. *Cell death and differentiation*, 6, 99-104.

Prestera, T., Holtzclaw, W.D., Zhang, Y. and Talalay, P. (1993) Chemical and molecular regulation of enzymes that detoxify carcinogens. *Proc. Natl. Acad. Sci. USA*., 90, 2965-2969.

Raitt, D.C., Johnson, A.L., Erkine, A.M., Makino, K., Morgan, B., Gross, D.S. and Johnston, L.H. (2000) The Skn1 response regulator of *Saccharomyces cerevisiae* interacts with Hsf1 in vivo and is required for the induction of heat shock genes by oxidative stress. *Molecular Biology of the Cell*, 11, 2335-2347.

Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1997) Antioxidant properties of phenolic compounds. *Trend in plant science*, 2(4), 152-159.

Roemer, T. and Bussey, H. (1991) Yeast β-glucan systhesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. *Proc. Natl. Acad. Sci. USA*. 88, 11295-11299.

Roemer, T., Delaney, S. and Bussey, H. (1993) *SKN1* and *KRE6* define a pair of functional homologs encoding putative membrane proteins involved in β-glucan synthesis. *Molecular and Cellular Biology*, 13(7), 4039-4048.

Roemer, T., Paravicini, G., Payton, M.A. and Bussey H. (1994) Characterization of the yeast (1→6)-β-glucan biosynthetic components, Kre6p and Skn1p, and genetic interactions between the *PKC1* pathway and extracellular matrix assembly. *The Journal of Cell Biology*, 127(2), 567-579.

Rushmore, T.H., Morton, M.R. and Pickett, C.B. (1991) The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *The journal of biological chemistry*, 266(18), 11632-11639.

Santoro, N. And Thiele, D.J. (1997) Oxidative stress response in yeast *Saccharomyces cerevisiae*. In Hohmann, S. and Mager, W.H. (eds.). *Molecular Biology Intelligence unit: Yeast stress responses*. Texas: R.G Landes company, 171-211.

Sen, S. and Chakraborty, R. (2011) The role of antioxidants in human health. *American Chemical Society* [online], Available from: http://pubs.acs.org [accessed 12 March 2012].

Sies, H. (1993) Strategies of antioxidant defense. *Eur. J. Biochem*., 215, 213-219.

Sies, H. (1997) Oxidative stress: oxidants and antioxidants. *Experimental Physiology*, 82, 291-295.

Steffen, R. (2002) *LacZ* assays in yeast. In Guthric, C. and Fink, G.R. (eds.). *Guide to yeast genetics and molecular and cell biology Part B, Method in Enzymology vol. 350*. San Diego: Academic Press, 112-131.

Stephen, D.W., Rivers, S.T. and Jamieson, D.J. (1995) The role of the *YAP1* and *YAP2* genes in the regulation of the adaptive oxidative stress responses of *Saccharomyces cerevisiae*. *Molecular Microbiology*, 16, 415-423.

Stephen, D.W.S. and Jamieson, D.J. (1996) Glutathione is an important molecule in the yeast *Saccharomyces cerevisiae*, *FEMS Microbiology Letters*, 141, 207-212.

Sunnarborg, S.W., Miller, S.P., Unnikrishnan, I. and LaPorte, D.C. (2001) Expression of the yeast glycogen phosphorylase gene is regulated by stress-response elements and by the HOG MAP kinase pathway. *Yeast*, 18, 1505-1514.

Toledano, M.B., Delaunay, A., Biteau, B., Spector, D. and Azevedo, D. (2010) Oxidative stress responses in yeast. In Hohmann, S. and Mager, W.H. (eds.). *Topics in current genetics 1 Yeast stress responses*. Germany: Springer-Verlag Berlin Heidelberg, 241-303.

Verhagen, H., Schilderman, P.A.E.L. and Kleinjans, J.C.S. (1991) Butylated hydroxyanisole in perspective. *Chem.-Biol. Interactions*, 80, 109-134.

WHO (1987) Food additives series 21: 608 Butylated hydroxyanisole [online]. Available from: http://www.inchem.org/documents/jecfa/jecmono/v21je02.htm [accessed 16 March 2012].

WHO (1999) Food additives series 42: 949 Butylated hydroxyanisole [online]. Available from: http://www.inchem.org/documents/jecfa/jecmono/v042je23.htm [accessed 16 March 2012].

Williams, G.M., Iatropoulos, M.J. and Whysner, J. (1999) Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives. *Food and chemical toxicology*, 37, 1027-1038.

Winston, F. (2008). EMS and UV mutagenesis in yeast. *Current Protocols in Molecular Biology* [online], April 2008, 13.3B.1-13.3B.5, available from: doi: 10.1002/0471142727.mb1303bs82 [accessed 16 June 2013].

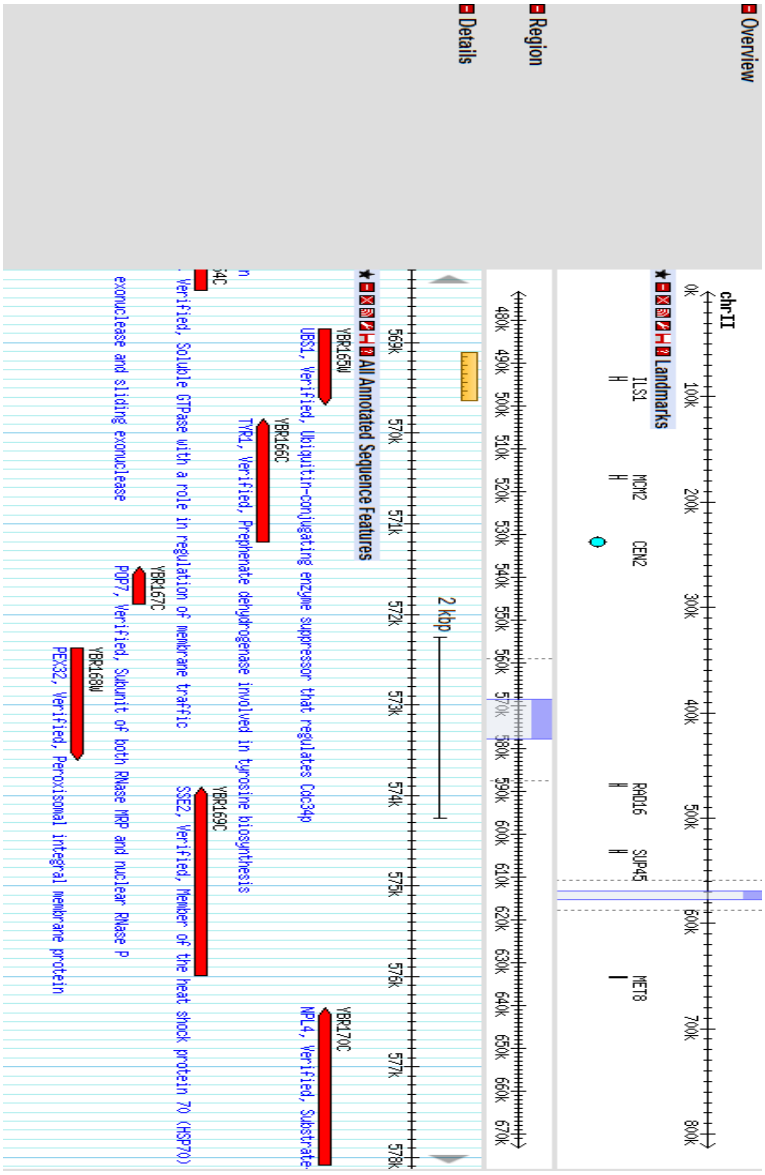
Yu, R., Mandlekar, S., and Kong, A.N.T. (2000) Molecular mechanisms of butylated hydroxylanisole-induced toxicity: Induction of apoptosis through direct release of cytochrome c. *Molecular Pharmacology*, 58, 431-437.

Zhang, Z., Moo-Young, M. and Chisti, Y., 1996. Plasmid stability in recombinant *Saccharomyces cerevisiae*. *Biotechnology Advances*, 14 (4), 401-435.

APPENDIX

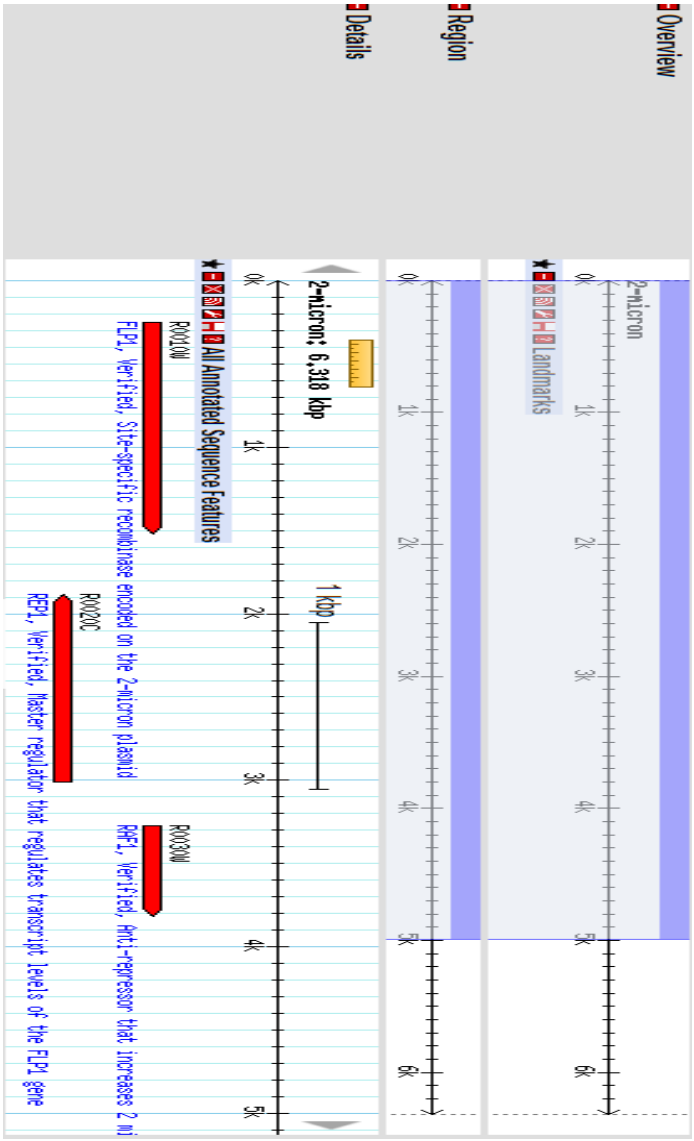
Appendix A. Maps of genomic DNA fragment in the complementing plasmid pYCC1, pYCC3, pYCC5, pYCC6 and pYQZ

The DNA insert of each complementing plasmid was sequenced and compared with *Saccharomyces* genome database (SGD) by performing BLAST searches. The genomic maps of DNA insert of each complementing plasmids are shown below.



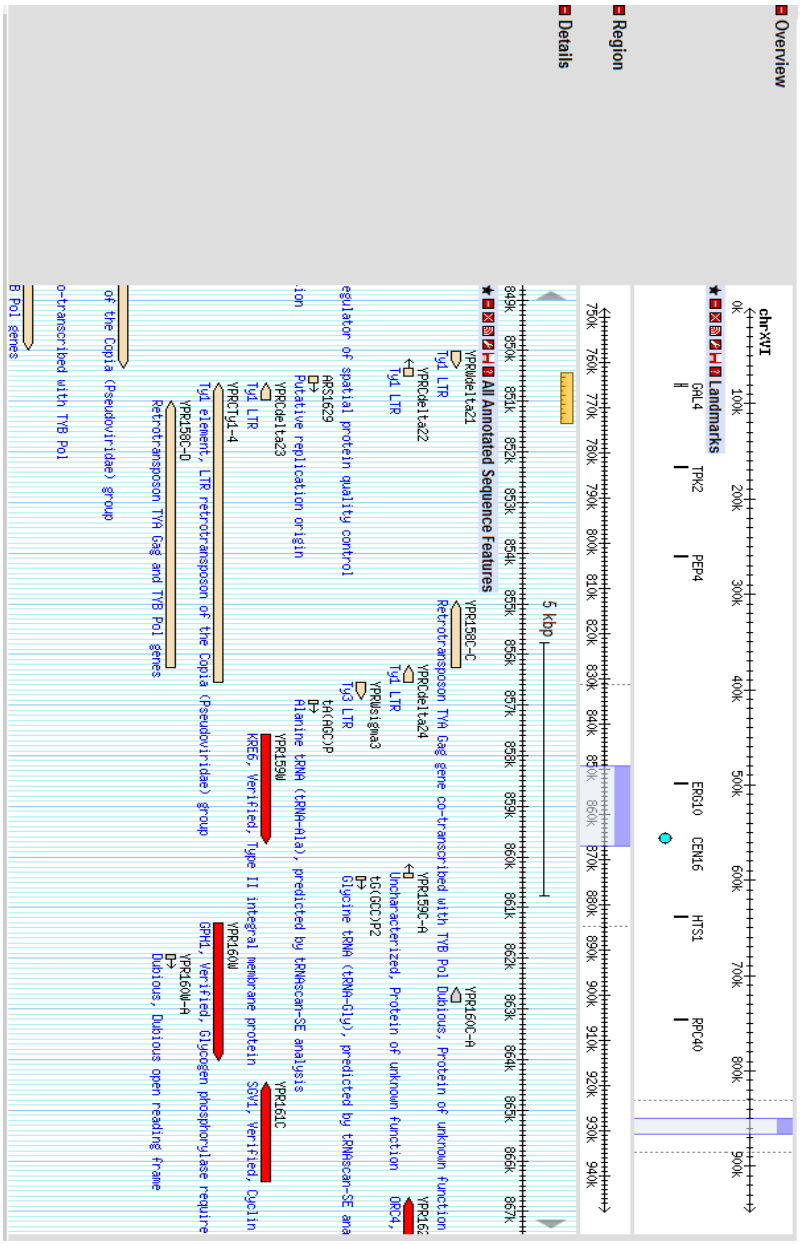
Source: www.yeastgenome.org

Figure 7.1 The genomic map of the DNA insert in plasmid pYCC1. The boundaries of the insert DNA is from 568433 bp to 577895 bp of Chromosome II.



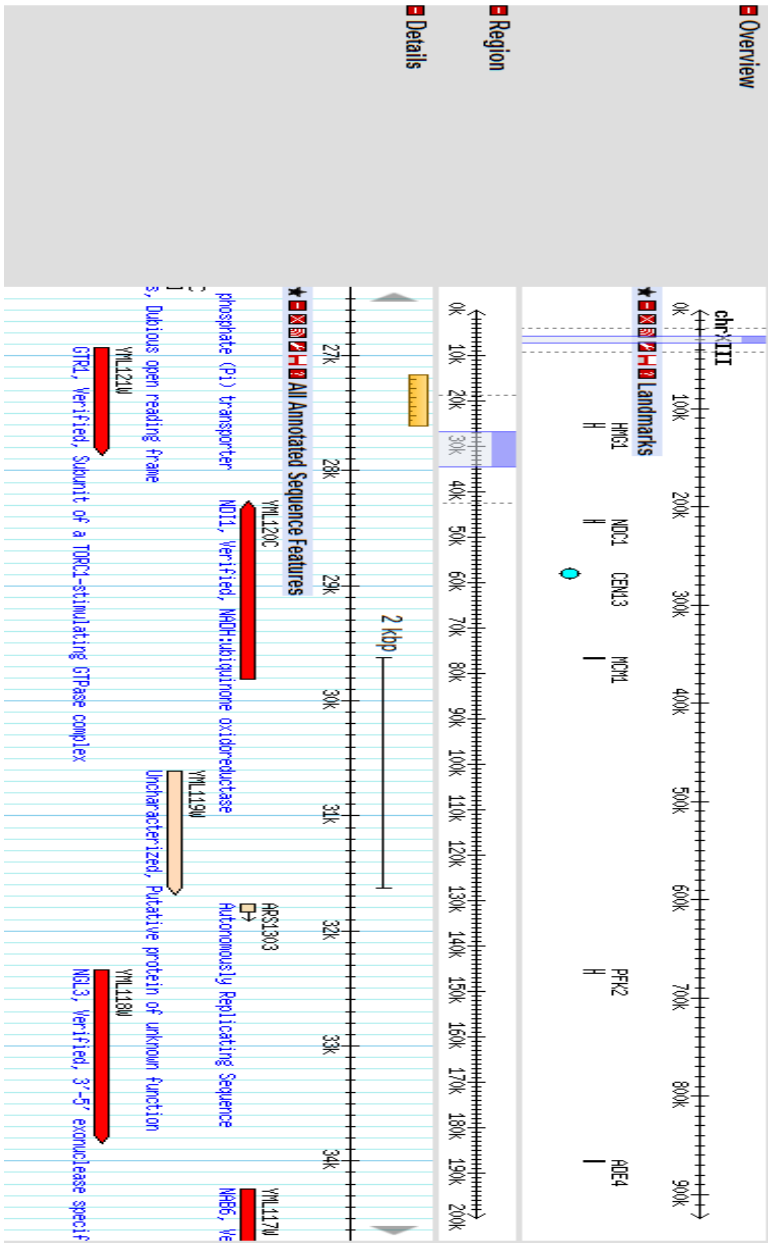
Source: www.yeastgenome.org

Figure 7.2 The genomic map of the DNA insert in plasmid pYCC3. The boundaries of the insert DNA is from 1 bp to 5000 bp of the 2-micron yeast plasmid.



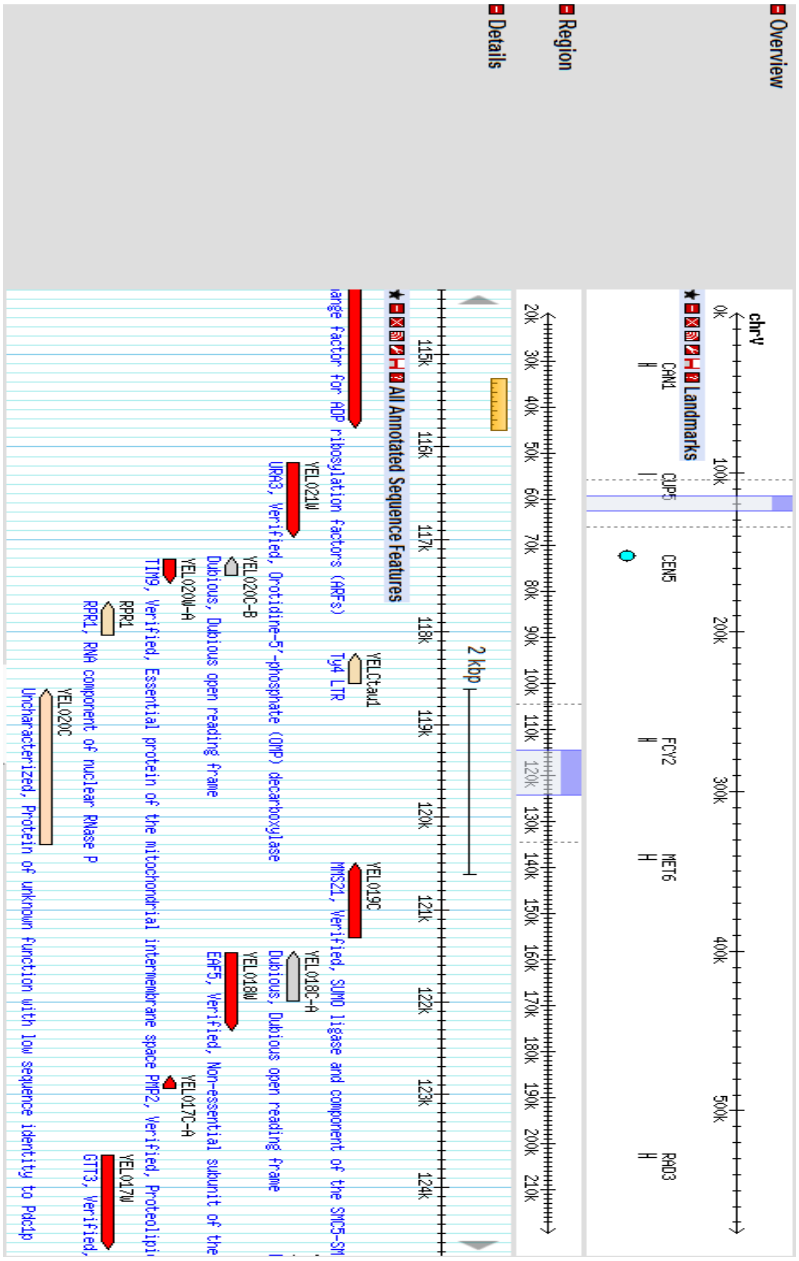
Source: www.yeastgenome.org

Figure 7.3 The genomic map of the DNA insert in plasmid pYCC5. The boundaries of the insert DNA is from 849164 bp to 867014 bp on Chromosome XVI.



Source: www.yeastgenome.org

Figure 7.4 The genomic map of the DNA insert in plasmid pYCC6. The boundaries of the insert DNA is from 26603 bp to 34500 bp on Chromosome XIII.



Source: www.yeastgenome.org

Figure 7.5 The genomic map of the DNA insert in plasmid pYQZ. The boundaries of the insert DNA is from 114551 bp to 124550 bp on Chromosome V.

Appendix B. Analysis of digested plasmids

The complementing plasmid pYCC1, pYCC2, pYCC3, pYCC4, pYCC5, pYCC6 and pYQZ were analyzed by agarose gel electrophoresis. Reaction of plasmid digestion was described in section 2.12. Visualization of these digested plasmids is shown in figure 7.6 and 7.7.

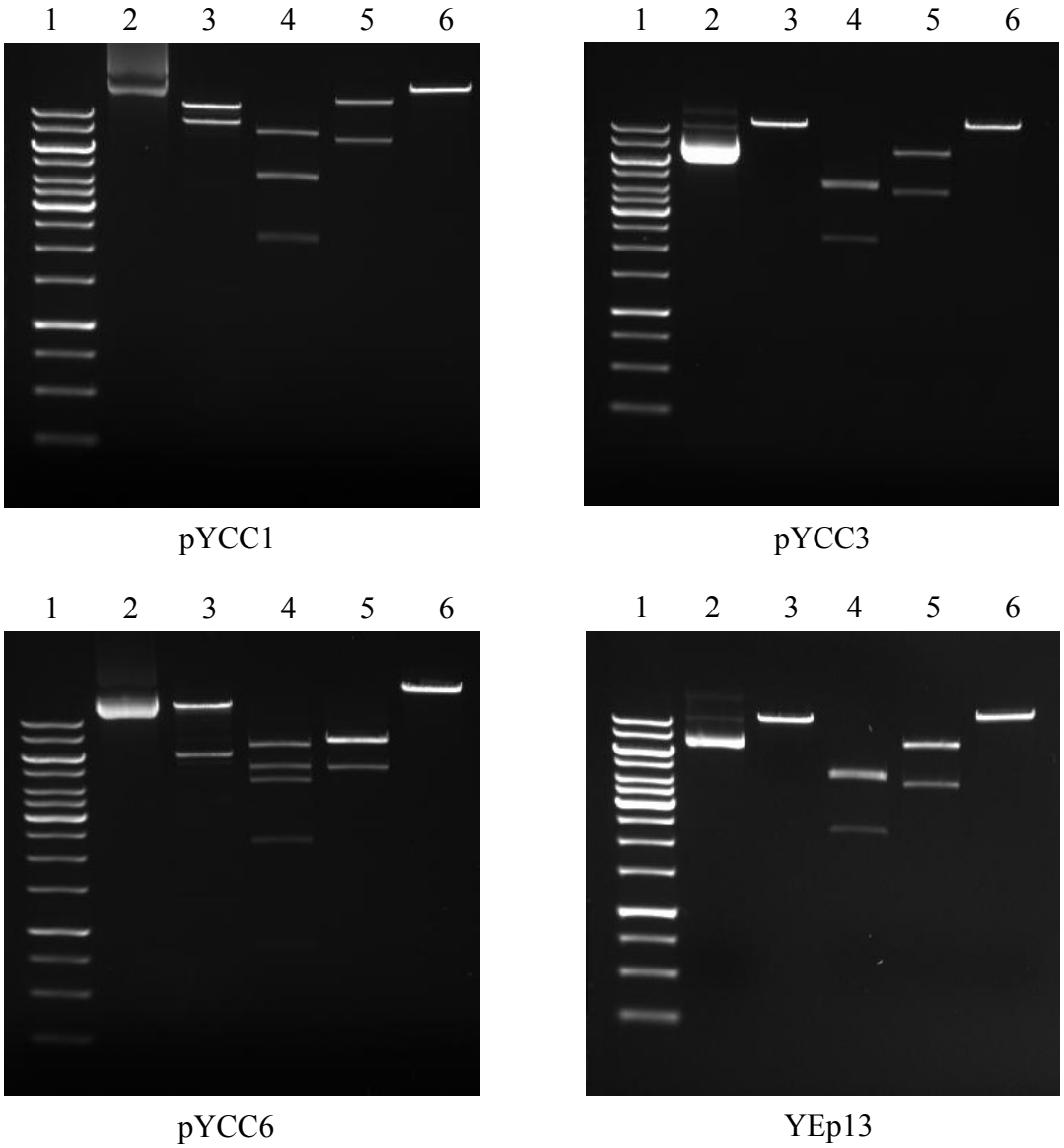
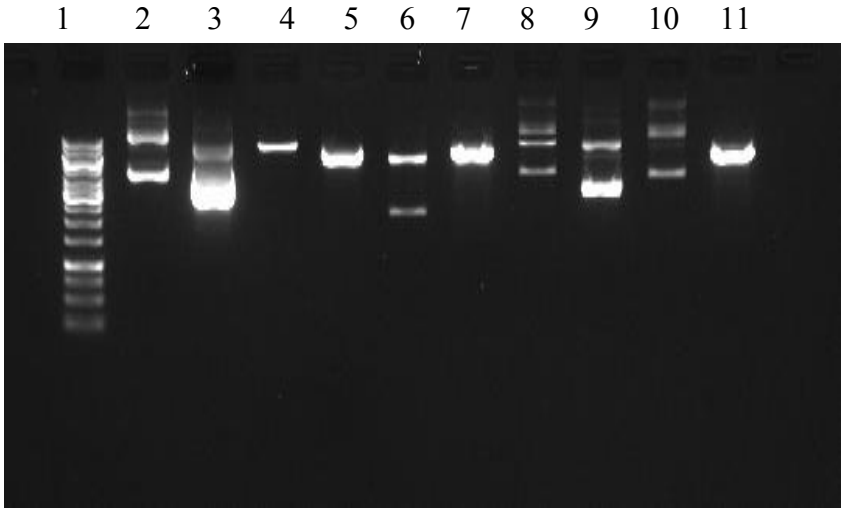
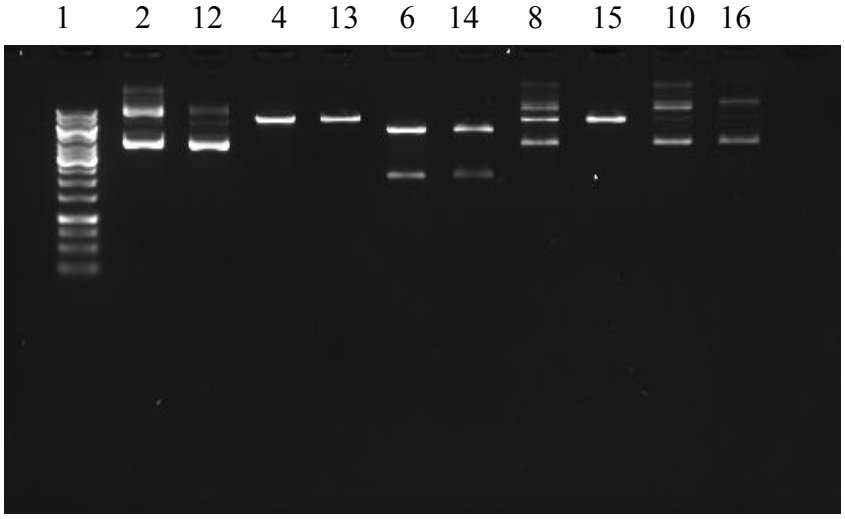


Figure 7.6 Example of visualizing digested plasmid pYCC1, pYCC3, pYCC6 and YEp13 (plasmid background) in agarose gel. Plasmid DNA was electrophoresed in 0.8% agarose gel for 45 minutes at 45 mV. The positions in the gel represent 1kb marker DNA (1), circular DNA (2), digested plasmid DNA with *Bam*HI (3), *Eco*RI (4), *Sal*I (5) and *Xho*I (6).



pYCC5



pYQZ

Figure 7.7 Visualizing digested plasmid pYCC5, pYQZ and YEp24 (plasmid background) in agarose gel. Plasmid DNA was electrophoresed in 0.8% agarose gel for 45 minutes at 45 mV. The positions in the gel represent 1kb marker DNA (1), circular DNA YEp24(2), circular DNA pYCC5 (3), digested YEp24 with *Bam*HI (4), digested pYCC5 with *Bam*HI (5), digested YEp24 with *Eco*RI (6), digested pYCC5 with *Eco*RI (7), digested YEp24 with *Sal*I (8), digested pYCC5 with *Sal*I (9), digested YEp24 with *Xho*I (10), digested pYCC5 with *Xho*I (11), circular DNA pYQZ (12), digested pYQZ with *Bam*HI (13), digested pYQZ with *Eco*RI (14), digested pYQZ with *Sal*I (15) and digested pYQZ with *Xho*I (16).